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Targeted Nanoparticles for Image-guided Treatment of Triple Negative Breast Cancer: Clinical Significance and Technological Advances

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Abstract

Effective treatment of triple negative breast cancer (TNBC) with its aggressive tumor biology, highly heterogeneous tumor cells, and poor prognosis requires an integrated therapeutic approach that addresses critical issues in cancer therapy. Multifunctional nanoparticles with the abilities of targeted drug delivery and non-invasive imaging for monitoring drug delivery and responses to therapy, such as theranostic nanoparticles, hold great promise towards the development of novel therapeutic approaches for the treatment of TNBC using a single therapeutic platform. The biological and pathological characteristics of TNBC provide insight into several potential molecular targets for current and future nanoparticle based therapeutics. Extensive tumor stroma, highly proliferative cells, and a high rate of drug-resistance are all barriers that must be appropriately addressed in order for these nanotherapeutic platforms to be effective. Utilization of the enhanced permeability and retention (EPR) effect coupled with active targeting of cell surface receptors expressed by TNBC cells, and tumor associated endothelial cells, stromal fibroblasts and macrophages is likely to overcome such barriers to facilitate more effective drug delivery. An in depth summary of current studies investigating targeted nanoparticles in preclinical TNBC mouse and human xenograft models is presented. This review aims to outline the current status of nanotherapeutic options for TNBC patients, identification of promising molecular targets, challenges associated with the development of targeted nanotherapeutics, the research done by our group as well as others and future perspectives on the nanomedicine field and ways to translate current preclinical studies into the clinic.

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Introduction

Despite extensive research and clinical strides being made, about 40,000 women in the U.S. alone are expected to die annually of breast cancer [1]. While mortality rates have decreased by 14% since 2008, the incidence of breast cancer worldwide has increased by more than 20% with nearly 1.5 million new cases each year [2, 3]. Molecular analysis of breast cancer tissues revealed the presence of four breast cancer subtypes, including hormone receptor (estrogen (ER) and progesterone (PR)) positive, human epidermal growth factor receptor 2 (HER-2) positive, triple negative (ER, PR and HER-2 negative), and normal breast [4–6]. At present, targeted therapies for hormone and HER-2 receptor positive cancers, such as the estrogen receptor antagonist (Tamoxifen) and anti-HER-2 antibodies (Trastuzumab and Pertuzumab), have been used in the clinic for breast cancer treatment [7, 8]. However, for triple negative breast cancer (TNBC) there are no such targeted treatments currently available highlighting not only the disparity in therapeutic options for these patients but also demonstrating an urgent clinical need towards the development of more personalized therapeutics.

TNBC, which accounts for 15–20% of all breast cancer cases, is among the most aggressive forms of breast cancer and is diagnosed more frequently in young African-American and Hispanic women [9, 10], and those with BRCA-1 mutations [11]. TNBC is diagnosed based on the absence of the ER and PR receptors and the lack of HER-2 overexpression of biopsied tumor samples by immunohistochemical (IHC) and fluorescence *in situ* hybridization (FISH) analyses. Due to its highly aggressive biology, the standard methods of detection, mammograms, magnetic resonance imaging (MRI) and ultrasound, typically detect TNBCs at later stages with large tumor lesions (>2.5 cm) and locally advanced disease [12]. In order to reduce large tumor burden, thus mediating complete surgical resection of the tumor, and to treat potentially disseminated tumor cells in distant organs, TNBC patients usually receive preoperative neoadjuvant chemotherapy, typically consisting of taxanes and anthracyclines [13]. However, over 30% of those TNBC patients develop local and distant recurrent tumors in visceral sites including the lungs, liver and brain and have a worse survival rate compared to non-TNBC, particularly within the first 5 years of diagnosis [14–16]. While TNBC patients have a poorer distant metastasis-free rate (67%) compared with non-TNBC patients (82%) during the first 5 years post diagnosis [17], the risk for recurrence after five years is decreased in TNBC patients compared with non-TNBC patients [14].

While generally successful at reducing the size of primary TNBC tumors, neoadjuvant chemotherapy provides an opportunity to determine the chemosensitivity of the tumors, which serves not only as a potential guide for future treatment of recurrent tumors but may also provide insight into overall survival benefit [15]. Results of clinical studies showed that TNBC patients have differential responses to chemotherapeutics and a population of TNBC patients (20 to 30%) tends to have increased pathological complete responses (pCR) relative to non-TNBC patients following neoadjuvant therapy and a better overall survival [15]. However, ~60% of TNBC patients have tumors highly resistant to chemotherapy. TNBC patients with BRCA1 mutations are sensitive to platinum agents and relatively less sensitivity to taxanes [18, 19]

Currently, there is no predictive biomarker to distinguish between the patients who will have good therapeutic responses and those patients who will not benefit from neoadjuvant therapy. The presence of large, drug-resistant residual tumors after neoadjuvant therapy has been associated with higher tumor recurrence and poorer survival [15]. Histological analysis of tumors of chemo-resistant patients revealed that a high level of drug resistant tumor cells express breast cancer stem-like cell biomarkers, $CD44^{hi}/CD24^{lo}$ [20]. Overall, TNBC patients that fail to achieve a pCR following neoadjuvant therapy have a worse prognosis compared to the patients with pCR [15, 21]. Therefore, the development of novel approaches to address two major challenges in the clinical management of TNBC patients, timely monitoring of therapeutic responses and effective treatment of drug resistant tumor cells, should have significant impact on the improvement of survival of TNBC patients.

The biology of TNBC and the clinical responses observed among TNBC patients is further complicated by the high degree of intratumoral (heterogeneity within a tumor) and intertumoral (heterogeneity within a given tumor) heterogeneity. While the majority of TNBC has a molecular gene signature associated with the basal-like subtype of breast cancer (BLBC), the remaining 20–30% of TNBCs are classified as other subtypes [22]. Lehmann *et al* [23] identified seven subtypes of TNBC based on global gene expression analysis: basal 1 (BL1), basal 2 (BL2), immunomodulatory (IM), luminal androgen receptor (LAR), mesenchymal (M), mesenchymal stem cell (MSL) and unstable (UNS). Each subtype has distinct characteristics which not only make them more sensitive to specific classes of drugs and inhibitors (Table 1) but is being found to serve as potential predictors of clinical responses following current therapy.

Retrospective studies are revealing that the differential chemotherapy responses are correlative with the seven TNBC subtypes. Masuda *et al* [24] reported that among 130 TNBC patients who had received standard neodjuvant chemotherapy (doxorubicin/cyclophosphamide/paclitaxel), the BL1 subtype had the highest pCR (52%) whereas LAR and BL2 had the lowest pCR with 10% and 0% respectively. Importantly, their findings indicated that TNBC subtype is an independent predictor of pCR status. They hypothesize that the differential responses between the BL1 and BL2 subtypes could be due to the enhanced gene signature of EGFR and IGF1-R pathways among the BL2 subtype which could in turn be utilized for the development of targeted therapies.

These classifications further demonstrate the advantages of stratifying/grouping patients based not solely on subtype but also by a defined set of biomarkers. Prat *et al* [25] recently identified that complete pathological responses (pCR) and improved survival after chemotherapy was associated with a proliferation signature or low expression of the luminal A signature among BLBC, not TNBC as a whole (Table 2). As more knowledge is gleaned about the various subtypes, more appropriate, personally tailored therapies can be developed and evaluated clinically.

The unique differential response to chemotherapy within the TNBC patient population makes it crucial to assess early tumor response to a given chemotherapy so that the patient will receive the most effective chemotherapeutics while avoiding unnecessary toxicity from an ineffective drug. Recent advances in the development of multifunctional nanoparticles

with the ability of targeted drug delivery and non-invasive imaging of biomarker expression, drug delivery and tumor responses (theranostic nanoparticles) offer great opportunities for novel, precision nanomedicine protocols to address the clinical challenges observed in TNBC treatment.

The development of targeted theranostic nanoparticles is highly significant for overcoming drug resistance of TNBC by: 1) targeted delivery of large doses of one or multiple drugs into cancer cells to maximize therapeutic effects while reducing systemic toxicity; 2) receptor targeted nanoparticles that promote intracellular drug delivery and release, and bypass multi-drug resistant protein (p-glycoprotein), located near the cell membrane, mediated efflux of drug molecules [26, 27] 3) capability of non-invasive imaging of intratumoral drug delivery and response that allows timely replacement of ineffective drugs to increase the pCR rate and overcome drug resistance; 4) systemic delivery of targeted theranostic nanoparticles that enables targeted therapy of locally disseminated tumor cells and distant tumor metastases, and 5) multimodal imaging ability of theranostic nanoparticles that provides imaging signals for intraoperative image-guided detection and removal of small drug resistant tumors to reduce local tumor recurrence.

Although non-targeted nanoparticles have been used in the clinic for cancer therapy, delivery of those nanoparticles is mediated by the enhanced permeability and retention (EPR) effect, which is primarily dependent on the tumor's leaky vasculature for entry and retention into the tumor bed [28, 29]. The EPR effect is often inefficient and provides minimal tumor specificity relative to normal organs. Identification of cell surface targets that are highly expressed in TNBC tissues should provide means for the development of targeted theranostic nanoparticles for effective treatment of TNBC patients.

Potential cellular molecules for the development of TNBC targeted theranostic nanoparticles

Based on the biology of TNBCs, potential molecular targets are being identified to mediate more efficient drug delivery as well as image-guided treatment. In comparison with other breast cancer subtypes, TNBC has unique pathological characteristics that need to be considered for the development of cancer therapeutics. There are very few cases of TNBC patients diagnosed at the early ductal carcinoma *in situ* stage [30]. The majority of TNBC tumors demonstrate aggressive behavior with high-grade tumour cells and a high percentage of proliferating tumor cells (Ki67 positive cells). The presence of extensive tumor stroma and infiltrating inflammatory cells is a marked pathological feature in TNBC tissues. A clinical study showed that 68% of TNBCs have tumors with over 50% consisting of intratumoral stroma and those patients have significantly higher tumor recurrent incidence and shorter survival time compared with the patients without enriched stroma [31]. It is well known that tumor stroma is one of the major barriers for drug delivery, especially for nanoparticle drug carriers. Therefore, it is important to consider the stromal affect when developing effective nanotherapeutics, for both targeted and non-targeted nanoparticles. It is likely that tumor targeting strategies enabling the nanoparticle carriers to efficiently extravasate and migrate through tumor stroma to reach tumor cells should offer promising targeted drug delivery approaches. Nanoparticles targeted to tumor cell surface targets alone

can only be delivered into the tumor interstitial space by “leaky” tumor vasculatures mediated by the enhanced permeability and retention (EPR) effect [32, 33]. Lack of mechanisms to overcome tumor stromal barriers will reduce the efficiency of tumor cell targeted nanoparticles to deliver the drug payload into tumor cells. Therefore, strategies that allow for active targeting to the tumor endothelial cell layer, tumor stroma as well as tumor cells should facilitate the drug-nanoparticles crossing the endothelial layer, in addition to the EPR effect of tumor vessels, thus increasing the overall efficiency of nanoparticle delivery into the tumor cells [34]. There are a number of molecules that are currently being investigated for the development of targeted therapies for TNBC based on their roles in tumorigenesis as well as their overexpression among breast cancer subtypes which are summarized in Table 3. Delivery mechanisms of different receptor targeted nanoparticles are shown in Figure 1. Although expression of these cellular surface targets is not specific for TNBC, a high level of the receptor expression in TNBC tissues supports the development of targeted nanoparticle drug carriers for effective treatment of TNBC.

Urokinase plasminogen activator receptor (uPAR)

In efforts to more effectively treat TNBC patients, targeting cell types found in the tumor microenvironment that contributes to the aggressive biology of this disease as well as tumor cells will likely prove most efficacious. uPAR, which plays a critical role in cell growth, motility and invasiveness [35], is overexpressed on the surface of many cellular components found within the tumor microenvironment including angiogenic tumor endothelial cells, stromal fibroblasts, and macrophages [36]. uPAR is detected in stromal macrophages of early breast lesions, which provides the opportunity for targeted delivery of imaging and therapeutic agents into small tumors [36]. The highest level of uPAR is usually found in the invasive edge of tumor cells and extensive tumor associated macrophages and fibroblasts [37]. The level of uPAR has been associated with a poor prognosis among breast cancer patients [38]. Therefore, uPAR targeted nanoparticle-drug delivery makes it possible to treat aggressive and invasive tumor cells as well as the stromal environment that promotes the invasiveness of TNBC tumor cells. Upon ligand binding, uPAR is internalized thus facilitating the uptake of the desired therapeutics. Several groups have developed uPAR or uPA targeted nanoparticle imaging probes or drug carriers for tumor imaging and targeted therapy using TNBC animal tumor models. uPAR targeted, dual optical and MR imaging probes have been shown to target primary and metastatic tumors following systemic delivery in the 4T1 TNBC mouse model and human TNBC tumor xenografts in nude mice [39, 40]. Radiolabelled antagonist antibodies targeting uPAR have proven successful in the reduction of TNBC tumor burden [41]. In addition to its attractive cellular distribution, uPAR expression has been shown to be correlative with a drug-resistant phenotype [42] which is highly prevalent among TNBC patients. LeBeau *et al* recently demonstrated successful targeting and imaging of uPAR by multiple imaging modalities including near-infrared (NIR) and single-photon emission computed tomography (SPECT) among drug-resistant TNBC xenografts [43].

Epidermal growth factor receptor (EGFR)

EGFR is a well-studied cell surface molecule that is overexpressed by TNBCs, which is generally more prevalent among TNBCs compared with non-TNBCs, with up to 50%

positivity [44, 45]. EGFR overexpression is associated with more aggressive, poorly differentiated tumors and is associated with a poorer clinical prognosis [46]. Phase II clinical trials indicated that EGFR-targeted monoclonal antibody cetuximab with the chemotherapeutic cisplatin extended progression-free survival and overall response rates among TNBC patients with metastatic diseases [47]. However, clinical investigation with small molecule EGFR inhibitor, erlotinib, has been disappointing with only 2 partial responses and no complete responses [48], which could be attributable to the lack of specificity of this class of tyrosine kinases and intrinsic resistance of TNBC cells [46]. However, overexpression of EGFR in TNBC tissues supports the potential development of EGFR targeted nanoparticles, imaging probes, and drug carriers that selectively deliver nanoparticles into EGFR expressing tumor cells to enhance imaging specificity and the effectiveness of cancer therapeutics. One drawback of EGFR targeted delivery of nanoparticles is that it relies on passive targeting to extravasate into the tumor interstitial space. However, due to the high level of expression, at a range of $7e^5$-$1e^6$ on the surface of each tumor cell [49, 50], EGFR is a viable target for nanoparticle delivery of therapeutics into tumor cells by receptor-mediated endocytosis.

Insulin growth factor 1 receptor (IGF-1R)

IGF-1R mediated signaling facilitates tumor cell growth, migration, invasion and survival [51] and is expressed at elevated levels on TNBC tissues relative to normal breast tissues [52] while being highly expressed in all subtypes of breast cancer [53, 54]. Results of preclinical studies demonstrated that IGF-1R targeting of TNBC lesions with dual-acting IGF-1R inhibitor BMS-754807 sensitized TNBC xenografts to chemotherapeutic apoptosis [55]. The use of IGF-1 for tumor targeting offers a natural ligand with high binding affinity and low immunogenicity. Due to receptor-mediated endocytosis, IGF-1 conjugated nanoparticles are less likely to deliver a stimulatory signal to IGF-1R expressing tumor cells. Lastly, IGF-1R is also highly expressed in drug-resistant TNBC [56], which further makes IGF-1R an appropriate target for TNBC.

Wnt pathway

Wnt signaling has been associated with stem cells and cancer stem-like cells. Activation of Wnt signaling enhances breast cancer cell motility and components of this pathway are up-regulated in TNBC [23, 57], whereas blockage of the Wnt pathway has been shown to inhibit cellular migration and induce apoptosis in TNBC cells [58]. Wnt signaling has also been associated with TNBC metastatic disease [59] and a lower disease-free survival rate [60]. Cellular receptors for Wnt ligands, such as Frizzled and lipoprotein receptor-related protein (LRP)-5/6, have been shown to mediate endocytosis [61]. Therefore, targeting Wnt receptors for efficient drug delivery into TNBC lesions can potentially enhance therapeutic responses specifically among drug-resistant, cancer stem-like cells.

Mucin 1 (MUC1)

The overexpression of the heterodimeric glycoprotein MUC1 is observed in over 90% of breast cancers with 67% of early-stage TNBC showing moderate to strong MUC1 expression [62]. While critical to the survival of normal epithelial cells, MUC1 overexpression protects tumor cells from stress-induced apoptosis [63]. The C-terminal subunit, MUC1-C,

interacts and forms complexes with receptor tyrosine kinases such as EGFR and HER-2 and contributes to their activation [64]. Direct targeting of MUC1 using the MUC1 inhibitors GO-201/203 was shown to markedly reduce the tumorigenicity of MDA-MB-231 [65] and MDA-MB-468 xenograft models of TNBC [66], thus making it an attractive target for nanoparticle-mediated delivery of therapeutic and imaging agents.

CD44

The adhesion/homing molecule CD44 is the primary surface receptor for hyaluronic acid (HA), a major component found in the extracellular matrix of most tissues but is overexpressed in tumor tissues [67]. HA synthesized by tumor and stromal cells is correlative with CD44 and HA synthase protein expression and is associated with more aggressive tumor behavior and poorer patient outcome [68]. Breast cancer stem cells, generally classified as $CD44^{hi}/CD24^{lo}$, contribute significantly to the drug-resistant phenotype observed among breast cancer patients. Likewise, these cells are found in a greater abundance in TNBC tissues relative to non-TNBC and are associated with a poorer prognosis [69]. Because CD44 can internalize HA [70], HA-conjugated nanocarriers are capable of delivering therapeutics specifically to CD44 over-expressing cells for enhanced efficacy [71].

Folate receptor

The folate receptor is an ideal and widely used protein for active targeting of drug delivery due to its overexpression by tumor tissues, with 86% of metastatic TNBC patients expressing this protein [72], as well as its limited normal tissue distribution on the apical surface of epithelial cells making it inaccessible to intravenously administered agents [73]. Similar to the before mentioned molecules, its expression is associated with a poor clinical prognosis among TNBC patients [74]. It has been shown that the folate receptor is expressed in inflammatory cells, tumor endothelial cells, and tumor cells and therefore, is a good candidate for the development of targeted therapeutics for TNBC. A number of folate receptor targeted imaging and therapeutic approaches have been developed and tested in pre-clinical and clinical trials [75].

CXCR4

Tumor expression of the chemokine receptor CXCR4 is associated with an aggressive phenotype, is up-regulated on metastatic tumors, and is correlative with a worse clinical outcome [76]. CXCR4 is also expressed at a high level in TNBC tissues [77]. Thus, active targeting of CXCR4 has the potential to enhance delivery of therapeutics to invasive and metastatic TNBC cells. Another appealing feature of targeting CXCR4 for the treatment of stroma-rich TNBC is that the receptor is also found in tumor-associated fibroblasts and macrophages [78]. Active tumor fibroblasts produce the ligand for CXCR4, stromal cell-derived factor1 (SDF-1), in order to promote tumor cell invasion and angiogenesis [79]. A small molecule CXCR4 antagonist (MSX-122) has been developed and its ability to specifically target primary and metastatic TNBC lesions has been demonstrated *in vitro* and *in vivo* in the MDA-MB-231 tumor xenograft model [80]. Such a small molecule ligand can be used for the development of targeted nanoparticle imaging probes and drug carriers.

Current Advances in Cancer Nanotechnology for TNBC

During the last decade, investigators in the nanomedicine field have developed various nanomaterials for the detection and treatment of breast cancer. Nanoparticles, typically between 1–1000 nm in size, can be made up of a variety of materials including lipids, polymers, silica, protein/peptides, oligonucleotides, and metals, such as gold, silver, and iron. Different types of nanoparticles provide unique chemical and physical properties for carrying therapeutic agents, intratumoral drug delivery, and tumor imaging [81].

At present, two nanoparticle formulated drugs have been used clinically for breast cancer treatment. The polyethyl glycosylated (PEG) liposome-encapsulated doxorubicin (Doxil) was determined to be more effective and less toxic when compared to conventional doxorubicin (Dox) [82, 83]. In 2005, the second nanoparticle drug was approved, nanoparticle albumin bound (nab)-paclitaxel (Abraxane) [84]. Nab-paclitaxel improved the anti-tumor activity and decreased toxicity when compared to solvent based paclitaxel in several types of human cancers, including breast cancer [85, 86].

The increased efficacy of both nanoparticle drugs is dependent on the principles that nanoparticle formulated drugs increase circulation time of the drug and can selectively deliver drugs into tumors by the EPR effect. Tumors undergo rapid vascularization which leads to impaired tumor vascular structures with “leaky” vessels and allows for nanoparticles smaller than 400 nm to accumulate in the tumor [32, 33]. Relatively large nanoparticle size compared to small molecule drugs minimizes the delivery of drugs into normal organs and tissues and therefore reduces systemic toxicity so that a higher drug dose can be administrated to cancer patients.

To overcome the clinical challenges for TNBC treatment, a broad range of nanoplatforms are under investigation as potential therapeutic options. Unique clinical and pathological properties of TNBC support the potential development of novel targeted and image-guided therapeutic approaches for the effective treatment of this aggressive type of breast cancer. To improve delivery efficiency of therapeutic agents into breast tumors, various nanoparticle drug carriers have been developed to target tumor cells, tumor vasculature, and the tumor microenvironment. The investigation of nanoparticles with the capacity for targeted drug delivery as an approach to treat metastatic disease, is of critical importance in TNBCs. Due to the increased likelihood of distant recurrence among TNBC patients, targeted theranostic nanoparticles has the unique potential to effectively detect and deliver chemotherapeutics to these metastatic lesions following systemic administration, based on the expression of the before-mentioned cellular receptors, especially receptors associated with tumor invasion and metastasis, such as uPAR and CXCR4. The effectiveness of these targeted drug carriers and imaging probes on drug delivery and tumor imaging have been demonstrated in multiple TNBC breast cancer cell lines and human xenograft tumor models as discussed herein.

Liposome Nanoparticles

Liposomal nanoparticles were the first nanoparticle drug carrier composed of a phospholipid bilayer. Liposomes possess a hydrophobic core which is ideal for encapsulating high concentrations of hydrophobic drugs and allows for controlled drug release [87]. Liposomes

coated with polyethylene glycol (PEG) decrease nonspecific uptake by macrophages in the reticuloendothelial system (RES), resulting in an increased blood half-life and bioavailability to tumors. Several non-targeted liposomal drug carriers have been developed for investigation in the treatment of TNBC in preclinical studies.

Systemic delivery of PEGylated liposome carrying a chemotherapeutic agent, arsenic oxide (AS_2O_3), referred to as arsenic nanobins, resulted in 3 to 5 fold increases in arsenic accumulation in tumors and enhanced antitumor effect relative to tumors treated with free AS_2O_3 in the MDA-MB-231 human breast cancer xenograft model [88]. Another non-targeted approach has been the development of pH sensitive liposomes that have the capacity to selectively release drug molecules into the acidic tumor environment or inside endosomes of tumor cells [89].

Endo-Tag-1 is a paclitaxel embedded liposomal nanoparticle that has been evaluated in a phase II clinical trial for advanced TNBC [90]. Its mechanism of action involves the negatively charged, newly formed tumor vasculature attracting the cationic liposome carrying the paclitaxel thus facilitating drug delivery. The paclitaxel can then attack the newly formed tumor vessels and cut off blood supply to the tumor. In a trial of 141 TNBC patients, at 16 weeks the disease free survival was 59.1% in the Endo-Tag-1/paclitaxel combination group compared to 48% in the paclitaxel only group [90].

Liposomes which are functionalized with monoclonal antibodies to specific target proteins, or immunoliposomes, can more effectively mediate intracellular drug delivery via receptor-mediated endocytosis. Cetuximab conjugated immunoliposomes carrying Dox showed strong tumor growth inhibition in the MDA-MB-231 xenograft model [91]. An anti-CXCR4 antibody conjugated and pH sensitive immunoliposome has been developed for the delivery of gene silencing small interference RNA (siRNA) for lipocalin-2 (Lcn2), a protein that is secreted by breast cancer cells and is associated with a poor prognosis [92]. CXCR4-Lcn2 siRNA-immunoliposomes significantly reduced cell motility in human TNBC cell lines but failed to inhibit cell viability.

Liposomes can also be targeted with small molecules and peptides. For example, folate receptor targeted PEGlyated poly(l-lactide) (PLLA) and poly(l-histidine) polymeric nanoparticles loaded with Dox showed tumor growth inhibition and reduced lung metastasis in the 4T1 murine mammary tumor model [93]. Therefore, targeted-liposomes are a promising nanoparticle platform in the treatment of TNBC disease.

To produce targeted liposomes with imaging ability, various imaging agents, such as organic dyes, gadolinium (Gd), radioisotopes and magnetic iron oxide nanoparticles (IONP), are either conjugated to or encapsulated within the liposomes [94]. Combining targeting and imaging offers a more effective approach for the treatment of TNBC using the liposome nanoparticles, allowing for the increased internalization of the drug payload into cells while allowing for imaging capabilities for monitoring drug delivery and therapeutic responses.

An octopeptide (Cys-Asp-Gly-Phe (3–5-DiF)-Gly-Ay-Cys-NH2) conjugated liposome targeting to α-integrin was loaded with NIR dye and dual therapeutic agents, Dox and rapamycin, a mTOR inhibitor [95]. Systemic delivery of this α-integrin targeted liposomal

carrier mediated selective accumulation of the nanoparticle-drug in MDA-MB-231 tumor xenografts, as observed by *in vivo* fluorescence imaging, and significantly decreased tumor volume compared to single agent treatment with either Dox or rapamycin. Another approach that has been taken by a number of research groups is to encapsulate or conjugate radionucleotides in addition to chemotherapeutic agents. EGFR targeted immunoliposomes labeled with technetium 99m (^{99m}T) were shown to be retained in the surgical cavity, had high accumulation in the residual tumor surface of MDA-MB-231 xenografts, and in the metastatic lymph nodes of nude rats by SPECT/CT imaging (Figure 2) [96].

Polymeric Nanoparticles

Polymeric nanoparticles are commonly used drug delivery vehicles that are biodegradable and have low toxicity. Many approaches have been developed to produce various polymeric nanoparticles by conjugation of multiple units of macromolecules or self-assembling of copolymers. Therapeutic agents can be encapsulated inside the nanoparticles or conjugated to polymers. Most polymeric nanoparticles were developed based on the poly (d,l lactidecoglycolide) PLGA polymer that has been approved by the FDA for therapeutic use in humans. Non-targeted polymeric nanoparticles drug carriers have been developed for preclinical investigations in TNBC tumor models including an active metabolite of irinotecan (SN38) encapsulated in polymeric nanoparticle that showed anti-tumor efficacy in the 4T1 mouse mammary tumor model [97]. Systemic delivery of IT-101, a camptothecin-conjugate cyclodextrin-based polymeric nanoparticle, showed a significantly stronger anti-tumor effect compared with conventional irinotecan in the human TNBC MDA-MB-231 model [98]. Currently, clinical trials are ongoing to determine the efficacy of IT-101 in cancer patients [99]. A PEGylated poly (epsilon-caprolactone)-carrying docetaxel (DTX) nanoparticle has also been shown to inhibit tumor growth and increase mouse survival compared to mice treated with conventional DTX [100]. The encapsulation of the water soluble platinum based agent, mitaplatin, in PLGA nanoparticles was also investigated and produced strong anti-tumor effects in nude mice bearing human TNBC xenografts derived from the MDA-MB-468 cell line [101]. Additionally, a cross-linked polymer cage that is sensitive to low pH was coated onto Dox-loaded liposomal nanobins and release of the payload drugs under the acidic tumor environments provided selective anti-tumor effect in the MDA-MB-231 TNBC xenograft model. Varying the degree of cross-linking in the polymer cage allows the surface potential to be fine-tuned for optimal stability, thus increasing circulation time and release properties [102]. As with liposomal nanoparticles, addition of targeting ligands and imaging agents has increased the effectiveness of polymer-based nanoparticles. Investigators have designed targeted polymeric nanoparticles with a diverse set of targets including cancer stem cell markers [103, 104], newly formed vasculature [105], and cell surface receptors [104–106].

An EGFR targeted peptide conjugated PLGA PEG polymeric nanoparticle has been developed for carrying dual chemotherapy drugs paclitaxel and aerobic glycolysis inhibitor, lonidamine. This polymeric nanoparticle showed targeted delivery into tumors and reduced systemic toxicity in an orthotopic, multidrug-resistant TNBC xenograft model [107]. Significant enhancement in therapeutic efficacy and altered multidrug resistance was observed within the EGFR-targeted nanoparticle treated group compared to the non-targeted nanoparticle treated group [108].

A hyaluronic acid (HA) conjugated, multi-layered nanoparticle targeting the CD44 receptor was developed consisting of multilayer polyelectric shell with one layer of polyanion HA and one layer of polycation L-lysine [109]. When the nanoparticle reaches an environment with a pH of 6.0, the nanoparticle expands from 17 nm to 53.2 nm, resulting in an increase in cellular uptake compared to control nanoparticles in MDA-MB-468 cell line and a 4-fold higher accumulation in MDA-MB-468 xenograft tumors compared to control non-targeted nanoparticles [109].

Various imaging agents have been incorporated onto targeted polymeric nanoparticles for monitoring drug delivery. NIR imaging has been extensively investigated due to its simplicity and capability for rapid real-time detection of nanoparticle-drug delivery and the potential for optical imaging of drug-resistant tumor cells for surgical resection. Huang *et al* designed a hyaluronic acid conjugated block copolymer (PLGA) that targets the CD44 receptor, encapsulates DTX as well as the NIR dye, DiR, into the nanoparticles (Figure 3) [110]. Systemic delivery of CD44 targeted-PLGA-nanoparticles led to effective accumulation of the nanoparticle in tumors in the MDA-MB-231 tumor xenograft model and facilitated NIR tumor imaging. The CD44 targeted-PLGA nanoparticle treated group also showed a marked decrease in tumor growth (92% growth inhibition) compared to the non-targeted group.

A multifunctional nanoparticle with potential for NIR imaging and phototherapy has also been developed. This nanoparticle was made from a poly (9,9—bis(4-(2-ethylhexylphenyl)-4,fluorine-alt-co-6,7-bis(4-(hexyloxy)phenyl)-4,9-di(thiophn-2-yl)-yhiadiazoloquinoxaline](PFTTQ) polymer that has a high NIR absorbance for infrared thermal images to be generated at the tumor site and upon irradiation at 808 nm for 5 min, the temperature can be raised to more than 50°C *in vitro* in MDA-MB-231 cells, resulting in tumor cell death [111]. Beyond polymer-based nanoparticles for NIR imaging, nanoparticles for clinically relevant imaging modalities such as PET and MRI have also been developed. An amphiphilic block copolymers poly (amide-amine)-poly (L-lactide)-b-poly ethylene glycol (PAMAM-PLA-PEG) nanoparticles that contained radiolabeled ^{64}Cu was developed (Figure 4) [112]. The nanoparticles were targeted to CD105, a protein expressed by neo-vasculature, by conjugating to the anti-CD105 antibody TR105. Serial non-invasive PET was used to measure PAMAM-PLA-b-PEG-TR105-^{64}Cu nanoparticle accumulation in the 4T1 murine mammary tumor model. Mice treated with PAMAM-PLA-b-PEG-TR105-^{64}Cu had a much higher level of nanoparticle accumulation according to PET imaging compared to non-targeted nanoparticles.

Carbon Nanotubes

Carbon nanotubes are cylindrical carbon nanostructures that are being investigated as drug delivery vehicles as well as imaging probes in TNBC. A PEGylated single-walled carbon nanotube (SWNT) conjugated with paclitaxel was shown to have a higher efficiency in suppressing tumor growth compared to conventional paclitaxel in the 4T1 mouse mammary tumor model [113]. SWNTs have intrinsic NIR photoluminescence and thus can be used for NIR optical imaging [114]. SWNTs with different ^{13}C/^{12}C isotope compositions and Raman peaks were synthesized and conjugation of different targeting ligands into those SWNTs

allowed for multiplexed Raman imaging of multiple biomarkers [115]. Strong optical absorbance of NIR light is the basis for photothermal cancer therapy. Systemic delivery of SWNTs into mice bearing 4T1 mouse mammary tumors led to NIR tumor imaging in the 1.0–1.4 μm emission region and tumor elimination based on photothermal effect at NIR 808 nm [116]. Complete tumor elimination was observed in photothermally-treated mice with no observed toxic side effects. SWNTs also produce excellent photoacoustic imaging contrasts for tumor imaging [116]. Compared to NIR optical imaging, photoacoustic imaging has a higher spatial resolution and deeper tissue imaging ability. However, to be able to translate this nanoparticle platform into future clinical translation, issues concerning long-term systemic and cellular toxicity, biodegradability, biodistribution and clearance of carbon nanotubes have yet to be investigated thoroughly.

Metallic Nanoparticles

Nanoparticles composed of metals or with metallic cores, such as gold and iron, have been used as drug carriers or theranostic agents.

Gold Nanoparticles—Several types of gold nanoparticles and nanorods have been developed as thermal therapeutic, imaging and drug delivery nanoparticles. A multilayered gold nanoparticle ($Au/SiO_2/Au$), referred to as a gold nanomatryoshkas, consists of a gold core coated with silica and a thin film of gold shell. Systemic delivery of nanomatryoshkas and irradiation significantly inhibited tumor growth and some mice were tumor free for over 60 days in a MDA-MB-231 xenograft model [117]. In addition to photothermal ablation therapy, gold nanoparticles can be loaded with chemotherapeutics to enhance antitumor efficacy. To treat metastatic breast cancer, a Dox loaded DNA wrapped gold nanorod was developed which allowed for dual therapeutic functions, photothermal ablation and chemotherapy [118]. Mice bearing 4T1 mammary tumors were treated with the Dox loaded DNA wrapped gold nanorod and received 655 nm laser irradiation. A significant reduction of primary tumor growth was observed in the gold nanorod treated mice as well as a suppression in lung metastases when compared to untreated mice [118].

Another hollow gold nanosphere which is a promising theranostic nanoparticle platform has plasmon absorption in the NIR region and displays strong photothermal coupling properties suitable for photothermal ablation therapy [119]. The hollow gold nanospheres (HAuNS, ~40-nm diameter) had the capacity to carry large amounts of Dox (63% by weight) and drug release can be triggered by NIR light irradiation. The dual therapeutic effects of Dox loaded-HAuNS and laser irradiation were demonstrated through enhanced cell death of combination treated groups compared to single treatment groups in the human TNBC MDA-MB-231 cell line [119].

The results from the studies described above demonstrate gold-based theranostic nanoparticles as an effective platform for the treatment of TNBC, especially in conjunction with photothermal ablation therapy. Questions still remain as to the biodegradability and clearance of gold nanoparticles in human subjects since cancer therapy requires large doses and repeated administrations, which may hinder the development of gold nanoparticles as clinically applicable theranostic agents. Further studies are required to elucidate the fate and

mechanisms of degradation and clearance of gold-based nanoparticles for future clinical translation.

Magnetic Iron-Oxide Nanoparticles—Magnetic iron oxide nanoparticle (IONP) is an attractive theranostic nanoparticle platform because of its capability as a drug carrier as well as a MRI contrast. IONPs are biocompatible and biodegradable nanoparticles with low toxicity. IONPs have unique paramagnetic properties, which generate a significant susceptibility effect resulting in strong T_2 and T^*_2 contrast, as well as T1 effect at very low concentrations [120]. Several forms of IONPs have been used in clinical settings and have proven to be safe for human use [121, 122].

MRI provides 3D anatomic resolution, soft tissue contrast, and unlimited tissue penetration depth. MRI is a common clinical imaging modality that makes it feasible to translate the MRI-guided cancer therapy into clinical applications. Several groups have developed targeted IONPs as imaging probes or theranostic nanoparticles [123]. IONPs targeting underglycosylated MUC-1 (uMUC-1) were developed by conjugation of MUC1 targeting peptides (EPPT) to NIR dye Cy5.5 labeled IONPs. This imaging IONP was used to monitor response of breast cancer to Dox treatment by MRI in a human TNBC BT20 cell line-derived xenograft model [124].

Our group has developed uPAR-targeted IONPs by conjugating a NIR dye labeled, recombinant amino terminal fragment (ATF) of mouse or human uPA to amphiphilic polymer coated IONPs [39, 125]. Systemic delivery of uPAR-targeted IONPs led to an accumulation of IONPs in tumors of Balb/c mice bearing 4T1 mouse mammary tumor or nude mice bearing MDA-MB-231 tumor xenografts and generated strong MRI T_2- contrast for tumor MRI [39]. We further demonstrated targeted delivery of NIR-dye labeled IONPs into mice bearing breast tumor xenografts enabling non-invasive multimodal tumor imaging by NIR optical, T_2-weighted or ultra-short TE MRI, 3D fluorescence tomography, and photoacoustic tomography [126–128] (Figure 5). uPAR-targeted nanoprobes significantly enhanced photoacoustic contrast of the tumor margins compared to non-targeted groups, with imaging to depths up to 31 mm. NIR-dye labeled uPAR targeted IONP was used for intraoperative optical imaging of tumor margins, allowing for complete removal of breast tumors [39]. Further, our *in vitro* data indicates that uPAR-targeted IONP-Dox deliver high levels of Dox into 4T1 and MDA-MB 231 cells and produce a strong inhibitory effect on cell growth when compared to cells treated with free Dox or non-targeted-IONP-Dox [125]. The ability of targeted therapy and MRI of nanoparticle–drug delivery following systemic delivery of uPAR-targeted IONP-Dox theranostic IONPs were demonstrated in 4T1 mouse mammary tumor model [129]. An intracellular adhesion molecule-1 (ICAM-1) antibody conjugated-IONP, developed by Guo *et al* [130] has been used as a MRI probe to evaluate tumor targeting in a TNBC xenograft model by MRI. The ICAM-1 targeted probe accumulated in ICAM-1 overexpressing TNBC tumor xenografts.

Due to the ability to assist in enhancing clinically relevant imaging modalities, such as MRI, a liposomal nanoparticle encapsulated with irinotecan (MM-398) in combination with an iron nanoparticle based imaging agent, ferumoxytol, has been used in a phase 1 clinical trial

in TNBC patients to assess the targeted drug into tumors and its relationship with the level of intratumoral macrophages[122].

Conclusion

Although extensive preclinical studies have been carried out in the development of numerous targeted nanoparticle imaging probes and drug carriers and evaluation of the effects of targeted tumor imaging and therapy, the process of translation of targeted nanoparticle agents into clinical applications has been challenging and relatively slow, compared with non-targeted nanoparticle drug carriers. One of the major issues is that many nanoparticle drug carriers targeted to cellular receptors are expressed only by tumor cells, such as EGFR and HER-2. Those targeted nanoparticles are delivered into the tumor using the same EPR effect mechanism as non-targeted nanoparticles. Nanoparticles targeted to tumor endothelial cells, such as RGD conjugated nanoparticles, target αVβ3 integrin in angiogenic tumor vessels but only a small percentage of human tumor cells express αVβ3 integrin. Following extravasation, the majority of targeted nanoparticle drug carriers were secluded in perivascular areas due to the presence of tumor stromal cells and extracellular matrix barriers[32]. Therefore without novel approaches to overcome tumor stromal drug delivery barriers, current methods for targeted delivery of nanoparticle drug carriers will fail to reach their fullest therapeutic potential for targeted cancer treatment. Success in translating targeted nanoparticles into clinical applications will require innovative nanoparticle designs to break tumor stroma and efficiently deliver nanoparticle-drug into tumor cells.

In this review, we narrowed our scope on experimental systems and results generated from TNBC cell line-derived animal models. Other theranostic nanoparticles and imaging approaches that have been developed and tested in other tumor types also have potential for targeted and image-guided treatment of TNBC. Additionally, the vast majority of preclinical studies on TNBC use the limited number of human, MDA-MB-231, MDA-MB-468, BT20, and 4T1 mouse mammary tumor cell lines and those cell line-derived xenograft TNBC tumor models in mice. However, due to the molecular heterogeneity of TNBC, novel imaging and therapeutic agents should be tested in models that more closely recapitulate human TNBC disease, such as patient derived xenograft (PDX) models.

Despite the observed preclinical efficacy of nanoparticles in TNBC models, in order to be translated into the clinics, several challenges remain: large scale production of consistent nanoparticle–drug carriers, improved delivery efficiency, new approaches to avoid liver and spleen nonspecific uptake, evaluation of pharmacokinetics and pharmacodynamics in preclinical studies, determination of systemic toxicity of targeted theranostic nanoparticles, the establishment of sensitive imaging methods and protocols for clinically available imaging devices as well as the development of new imaging devices for new types of theranostic nanoparticles. With the significant and promising progress in the delivery and imaging of nanocarriers to treat breast cancers, including TNBC, strides are being made toward the critically needed translational and clinical discoveries that are on the horizon.

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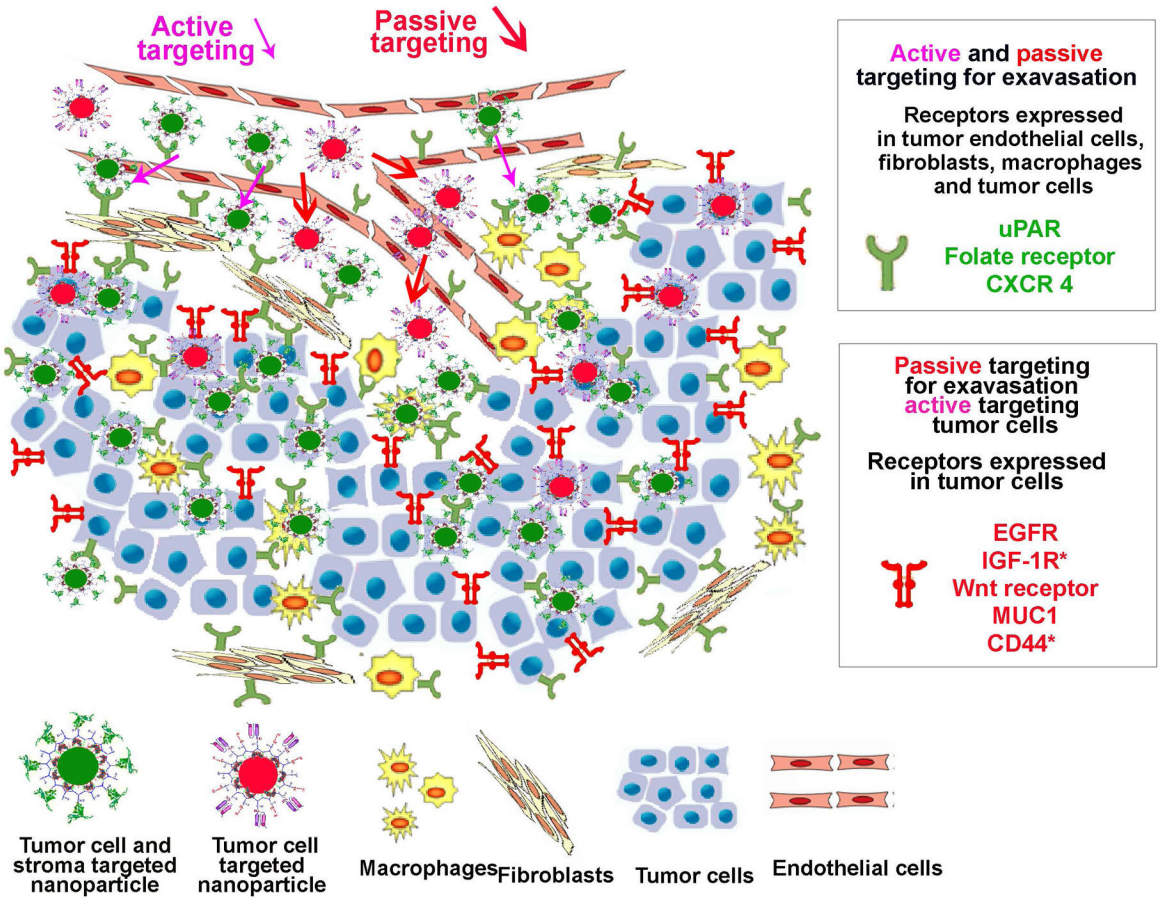

Figure 1. Cellular receptor highly expressed in TNBC tissues for the development of targeted theranostic nanoparticles

Cellular receptors upregulated in TNBC tissues have differential levels in tumor cells and stromal cells. uPAR, folate receptor and CXCR4 are expressed in tumor cells, angiogenic endothelial cells, and stromal fibroblasts and macrophages. EGFR, Wnt receptor and MUC1 are found in tumor cells. IGF-1R and CD44 are highly expressed in tumor cells and some stromal cells.

Tumor endothelial cell targeted theranostic nanoparticles are delivered into TNBC tissues by both active targeting and passive targeting (or EPR effect). Theranostic nanoparticles targeting to tumor cells alone are delivered by passive targeting into the tumor interstitial space. Receptor targeted theranostic nanoparticles with cellular targets expressed in tumor cells and stromal fibroblasts and macrophages, but lack the expression in tumor endothelial cells, will also be delivered into the tumor interstitial space by passive targeting. The binding of the targeted theranostic nanoparticles to stromal fibroblast, macrophages, and tumor cells enhances retention of the nanoparticles in the tumor. Receptor-mediated internalization of nanoparticle drug carriers increases intratumoral cell drug delivery and therapeutic effect.

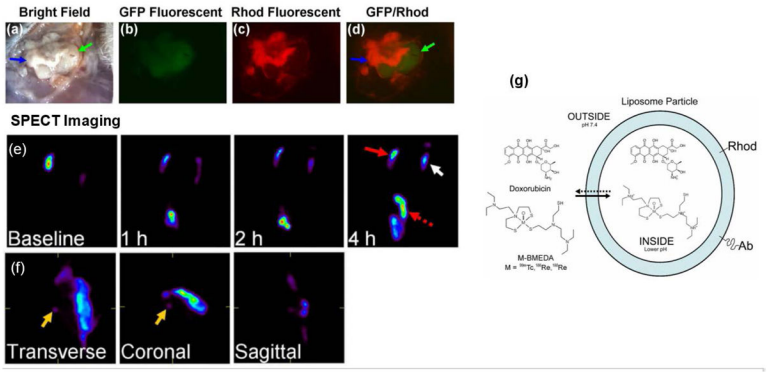

Figure 2. Receptor targeted radioactive immunoliposomes for SPECT imaging of residual breast tumors in lumpectomy cavity and draining lymph nodes

Stereomicroscopic fluorescent images (a–d) and SPECT images (e–f) of MDA-MB-231 rat xenograft injected with ^{99m}Tc labeled panitumumab (EGFR antibody)-liposomes containing RhodDOPE tracer. A schematic of the ^{99m}Tc labeled panitumumab liposomes (g). Reproduced with the permission from Molecular Pharmacology Online by American Society for Pharmacology and Experimental Therapeutics [96].

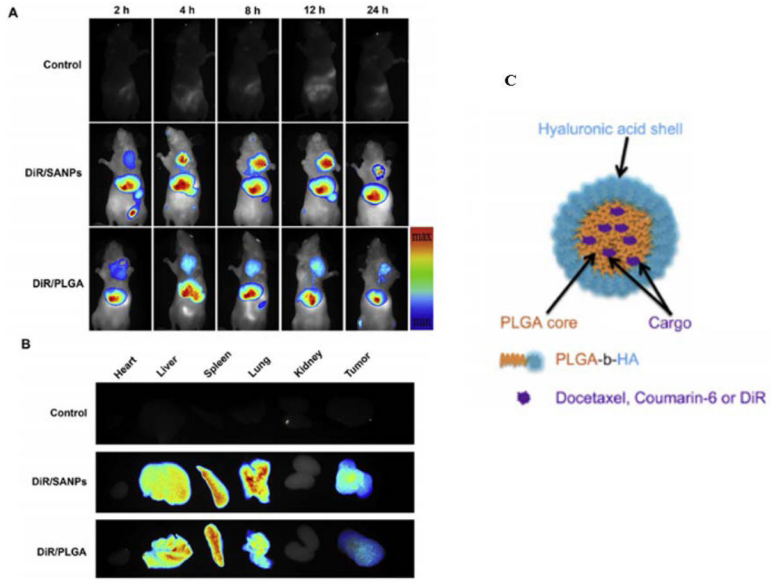

Figure 3. CD44 targeted polymeric nanoparticle carrying docetaxel for targeted delivery into TNBC tumors

Fluorescence images of DiR loaded PLGA502H (DiR/PLGA) and DiR loaded PLGA502H-b-HA5.6k nanoparticles (DiR/SANPs) in MDA-MB-231 tumor-bearing female nude mice after tail vein injection. (A) In vivo whole body images and distribution of nanoparticle formulations at varying time intervals. (B) Ex vivo images of excised organs and tumors at 24 h post-injection of the formulations. (C) The schematic illustration of the core-shell structure of docetaxel (DTX)-loaded PLGA-b-HA nanoparticles that target CD44. Reproduced with the permission from Biomaterial Journal and Elsevier publisher [110].

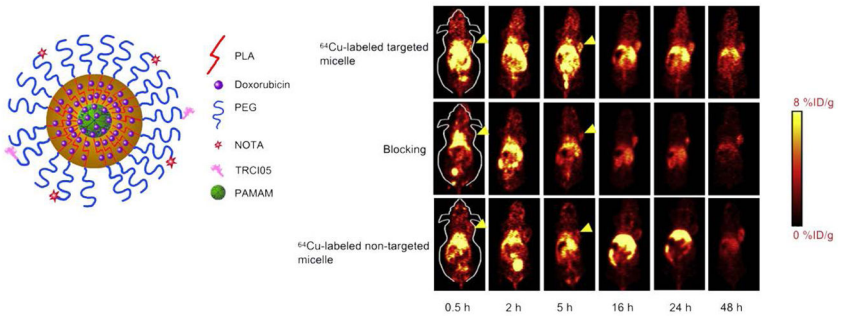

Figure 4. Angiogenic tumor vessel targeted and ^{64}Cu radiolabeled PAMAM-PLA-PEG theranostic nanoparticles for targeted therapy and PET imaging of TNBC

(A) Schematic illustration of the multifunctional PAMAM-PLA-b-PEG-OCH_3/TRC105/NOTA unimolecular micelles for tumor-targeted drug delivery and PET imaging. (B) Serial coronal PET images of 4T1 tumor-bearing mice at different time points post-injection of ^{64}Cu-labeled targeted micelles, non-targeted micelles, or targeted micelles with a blocking dose of TRC105. Reproduced with the permission from Biomaterial Journal and Elsevier publisher [112].

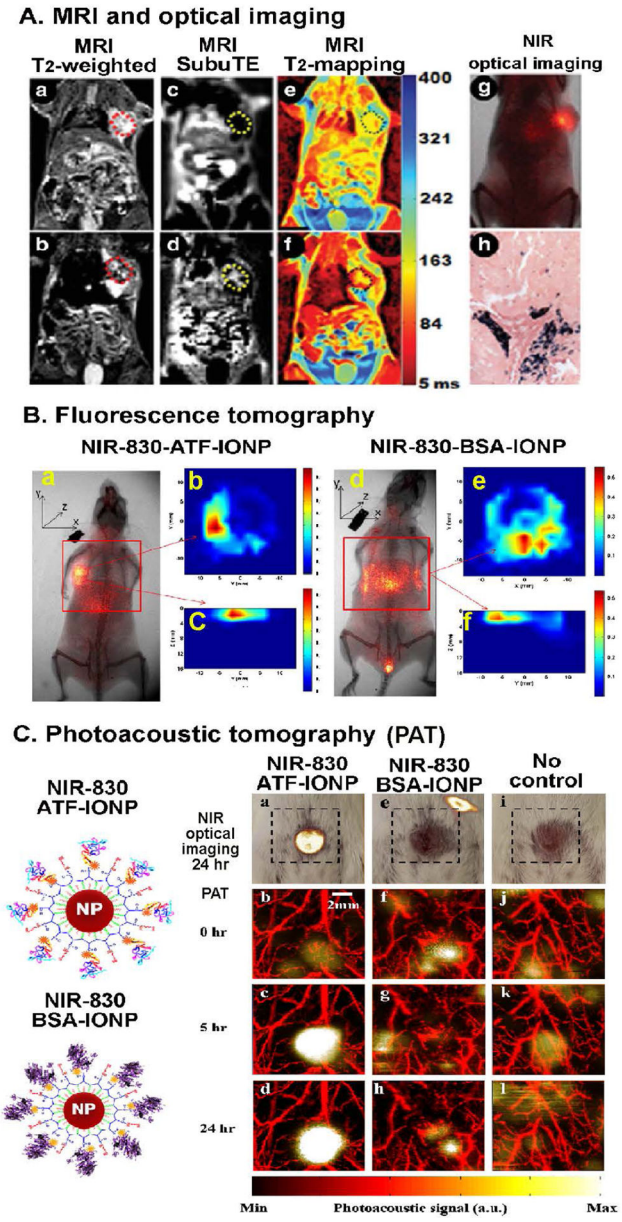

Figure 5. uPAR-targeted magnetic iron oxide nanoparticles for multimodal imaging of breast cancer

Balb/c mice bearing 4T1 mammary tumors (TNBC subtype) received a tail vein delivery of 300 pmol of NIR-830-dye-mouse ATF-IONP. **A**. Dual NIR optical and MR imaging. 24 hr following nanoparticle injection, MRI Signal decreased in T_2-weighted images (a,b). An ultrashort echo time (UTE) imaging detected an increase in MRI signal (positive MRI signal) shown as SubUTE image by subtraction of a longer echo signal from that of the UTE (SubUTE) image (c.d). T_2-maps showed T_2 signal decreases in the tumor. Orthotopic mammary tumor (dot lined-area), a, c, and e: before injection, b,d, and f. post injection. NIR optical imaging showed strong signal in a mammary tumor (g). Prussian blue staining of the tumor tissue section showed the presence of blue nanoparticle positive cells in the tumor (h). Reproduced and modified with the permission of John Wiley and Sons Inc [126]. **B.**

Targeted fluorescence tomography (FMT). NIR 830-ATF-IONP: uPAR-targeted nanoparticle, NIR-830-BSA-IONP: bovine serum albumin (BSA) conjugated nanoparticle as a non-targeted control. FMT detected strong optical signal in the mammary tumor of the mice that received uPAR-targeted ATF-IONP (a, b, c), but not non-targeted BSA-IONP, which only showed strong signal in the liver area (d, e, f). a and d: x ray/planar fluorescence image of the mice; b and e: cross section of the FMT slice; c, and f: sagittal FMT slice. The red square in (a and d) indicates the FMT imaging area. Reproduced and modified with the permission of the Optical Society [127]. **C**. Photoacoustic imaging (PAT). Schematics showed uPAR targeted (NIR-830-ATF-IONP) or non-targeted (NIR-830-BSA-IONP). *In vivo* PAT and fluorescence images showed before and after nanoparticle injection. Macrographs were merged with fluorescence images taken 24 hours post injection with NIR-830 dye labeled uPAR targeted (a, b, c, d) or non-targeted IONP (e, f, g, h). A tumor bearing mouse without nanoparticle injection was imaged as a control (i, j, k, l). Panel b through l: PAT images were merged with blood vessel images before injection (b, f. j), and at 5 hours (c, g, k) and 24 hours (d, h, i) post injection. Reproduced and modified with the permission of John Wiley and Sons Inc [128].

Table 1

TNBC subtypes and treatment responsiveness [23]

	Subtype	Characteristics	Treatment Sensitivity	Cell line derivative
Basal-like	Basal-like 1 (BL1)	Highly proliferative, DNA damage and cell cycle genes	Cisplatin; taxanes	HCC1806 and MDA-MB-468
	Basal-like 2 (BL2)	Growth factor signaling pathways	----	
	Immunomodulatory (IM)	Immune cell processes and signaling pathways	----	HCC1187 and DU4475
	Luminal androgen receptor (LAR)	Steroid synthesis, androgen/estrogen metabolism	mTOR inhibition; AR agonist bicalutamide	MDA-MB-453, SUM185PE, HCC2185, CAL-148, and MFM-223
	Mesenchymal (M)	Cell motility and differentiation pathways	PI3K/mTOR inhibitor (NVP-BEZ235)	----
	Mesenchymal-like (MSL)	Cell motility and differentiation pathways; growth factor signaling pathways, low levels of proliferation genes	PI3K/mTOR inhibitor (NVP-BEZ235); Chemoresistant	CAL-51 and SUM159PT

Abbreviations: mTOR: mammalian target of rapamycin, AR: androgen receptor, PI3K: phosphatidylinositol-3-kinase

Table 2

Biomarkers for better characterization of TNBC

Biomarker	Stage	Finding	Reference
Proliferation signature/low luminal A signature	Clinical	Association with better survival among BLBC	Prat et al 2014 [25]
BRCA1 gene	Clinical	BRCA1 loss of function relates to treatment sensitivity	Kennedy et al 2004[131]
Androgen receptor (AR)	Clinical	AR^+ BC less responsive to NAC	Masuda et al 2013 [24]
CD73	Preclinical	Poor prognosis, increased resistance to anthracyclines	Loi et al 2013 [132]

Table 3

Potential cell surface targets for enhanced drug delivery in TNBC

Protein	Cellular distribution	Role in TNBC tumorigenesis
uPAR	Tumor cells, tumor endothelial cells, stromal fibroblasts and macrophages	Motility, invasiveness, angiogenesis.
EGFR	Tumor cells	Cell proliferation, survival, EMT
IGF-1R	Tumor cells, stromal macrophages and fibroblasts	Cell growth, migration, survival
Wnt receptor	Tumor cells, cancer stem cells (CSCs)	Cell proliferation, survival, differentiation, motility
MUC1	Tumor cells	Confers resistance to apoptosis
CD44	Cancer stem cells (CSCs), stromal cells	Initiation/maintenance of CSCs
Folate Receptor	Tumor cells, tumor endothelial cells, stromal cells	Cell proliferation
CXCR4	Invasive tumor cells, tumor endothelial cells, stromal cells	Metastasis, stromal cell infiltration