**Investigate Current Anticancer Nanomedicine Design Principles and Identify New Criteria to Improve Clinical Efficacy and Safety**

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Investigate Current Anticancer Nanomedicine Design Principles and Identify New Criteria to Improve Clinical Efficacy and Safety

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TOC

Universal Nanoformulation
Three Design Principles:
- Tumor target by EPR
- Long circulation and high plasma concentration
- One nanoformulation for different drugs

Preclinical Evaluation
Delivery Efficiency/Efficacy in Xenograft Tumor

Clinical Translation
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- Intrinsic shortcomings of drug’s physicochemistry, PK, PD
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Preclinical Evaluation
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Clinical Translation
Efficacy vs. Adverse Events

Spontaneous Transgenic Cancer
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Human Cancer Patients
**ABSTRACT**

We investigated the three anticancer nanomedicine design principles and their clinical translations by evaluating the distinct clinical efficacy/safety of five clinically approved nanomedicines with different nano features (size, composition, structure, long/short circulation), and comparing to the delivery efficiency in three types of preclinical cancer models. Results showed that nanomedicines enhanced tumor accumulation (3 to 5-fold) by EPR in subcutaneous and orthotopic xenograft cancers, but decreased tumor accumulation in spontaneous transgenic cancers. The tumor delivery efficiency/efficacy of nanomedicines in preclinical xenograft cancers neither translated to clinical efficacy nor was consistent with EPR heterogeneity hypothesis. The three distinct efficacy/safety profiles of long-circulating nanomedicines (Doxil®, Myocet®) were associated with their unique tissue distributions/penetrations in the skin and heart. The four distinct efficacy/safety features of short-circulating nanomedicine (Abraxane®) were associated with its distinct tissue distributions/penetrations in the pancreas, lung, and breast, blood cells, and GI tract. Further, long systemic circulating nanomedicines, without adequate tumor accumulation by EPR, violates mass balance principles for improving anticancer efficacy by reducing drug tissue distribution/penetration. Our data suggest that nanomedicine design principles based on tumor EPR and long circulation are only valid in xenograft models, which exaggerates the delivery efficiency/efficacy with little clinical translation. It is not feasible to develop a universal nanoformulation based on the same design principle for different anticancer drugs with distinct properties. Rather, a drug-specific nanoformulation needs to be developed to overcome the intrinsic shortcomings of the delivered drug to improve clinical efficacy/safety, using three new criteria based on drug’s distinct physico-chemical, pharmacokinetic, pharmacodynamic properties.
**Introduction**

In the past few decades, hundreds of anticancer nanomedicines have been generated and tested to show outstanding anticancer efficacy advantages in comparison with free drugs in preclinical cancer models [1-9]. However, the frequent clinical failures of most anticancer nanomedicines, with a few exception, raised questions for the current anticancer nanomedicine design principles [1-3, 5, 10-21]. The most recent closure (2019) of NCI Centers of Cancer Nanotechnology Excellence (CCNEs) further fueled the debates for the future of nanomedicines [22-24]. It is not clear if current nanomedicine design principles hinders the realization of the true benefit of anticancer nanomedicines in cancer patients.

The current anticancer nanomedicine design principles have three components: (A) The nanomedicine increases drug accumulation in tumors by the enhanced permeability and retention (EPR) in the tumor [7-9] to improve anticancer efficacy [7, 25-27]. (B) Long systemic circulation of anticancer nanomedicines, which is achieved by modifying nanomedicines (e.g. PEGylation), reduces non-specific uptake by reticuloendothelial system (RES) in the liver and spleen, decreases drug accumulation in the normal organs to reduce toxicity, and further enhances EPR effect for drug tumor accumulation [28-29]. (C) A universal nano delivery system with the same design principle can be developed for different anticancer drugs to improve anticancer efficacy and reduce toxicity in normal organs.

The first nanomedicine design principle based on tumor EPR has been confirmed in the mouse subcutaneous xenograft cancer model [1-9]. However, the clinical translation of this principle in human cancer patients was heavily debated due to two obvious reasons. Frist, the clinical failure to improve anticancer efficacy of most failed nanomedicines invalidate EPR in human tumor. Unlike small molecules, nanomedicines are inherently trapped in the xenograft tumors through
EPR effect in the xenograft tumors. It is not clear if this is an “intrinsic property” only in the artificially implanted xenograft cancer, which grossly exaggerates delivery efficiency/anticancer efficacy with little or no clinical translation in cancer patients? Second, analysis of the all successful nanomedicines together showed that their distinct clinical efficacy/safety are inconsistent with tumor EPR hypothesis. For instance, both PEGylated liposome Doxil® (85 nm) and Non-PEGylated liposome Myocet® (180 nm) has similar three new unique features in comparison to doxorubicin: superior clinical efficacy to treat AIDS-related Kaposi’s sarcoma but no efficacy difference to treat breast, ovarian cancers, or myeloma; reduced cardiotoxicity; and increased hand-foot-syndrome. In contrast, Abraxane® (135 nm), which is albumin nanoparticle of Paclitaxel, has four unique features in comparison with paclitaxel (Taxol®): superior clinical efficacy in treating pancreatic cancer, non-small cell lung cancer, and metastatic breast cancer; decreased incidence of neutropenia; increased incidence of neuropathy; and increased incidence of gastrointestinal toxicity. Genexol-PM® (22 nm), which is PEG-PLA polymeric nanoparticle of paclitaxel, has two unique features: non-inferior efficacy in treating breast cancer and increased incidence of neutropenia. It is not clear what are the mechanisms for each of these nanomedicines to exhibit its unique efficacy/safety profile in human cancer patients.

To consolidate the discrepancy of delivery efficiency/efficacy between animal xenograft model and clinical efficacy, EPR heterogeneity was hypothesized in different types of human cancers or in the same type of cancer of different patients. It is hypothesized that AIDS-related Kaposi sarcoma (ARKS) has better EPR than other solid cancer (breast), which explains the superior efficacy Doxil® in ARKS, but not in other solid cancers (such as breast) in comparison with free doxorubicin. However, the contradicting evidence against heterogeneous EPR
hypothesis in human is that Abraxane had superior efficacy in the breast, lung, and pancreatic cancer in comparison with paclitaxel 63-66; while Genexol-PM also showed non-inferior (or superior) efficacy in breast cancer to paclitaxel58.

The second nanomedicine design principle is to achieve long systemic circulation and high plasma concentration, which is achieved by PEGylation of the surface of nanoparticles to reduce RES clearance from the liver and spleen. However, from mass balance perspective, it not known if long circulation nanomedicine design, without adequate tumor EPR in human tumors, may violate mass balance principle by decreasing tissue distribution/penetration, which in turn reducing both anticancer efficacy and toxicity. Further, it is not known if achieving high plasma concentration should be a nanomedicine design criteria, without knowing drug concentration in the targeted tissues, since high plasma concentration rarely correlates with clinical efficacy/safety profiles of anticancer nanomedicines. For instance, the extremely higher (100 to 1000-fold) plasma concentration of long circulating Doxil® is neither translated to better efficacy against breast cancer nor explains its reduced cardiotoxicity in comparison with doxorubicin 20-21, 30-34. In contrast, the lower plasma concentration (3 to 5-fold) of short circulating Abraxane® cannot explain its superior clinical efficacy in breast, lung and pancreatic cancer in comparison with paclitaxel 63-66. It is not clear what should be the criteria to design nanomedicines with long or short circulation to improve clinical efficacy/safety.

The third nanomedicine design principle is to develop a universal nano delivery system for different anticancer drugs to improve their efficacy and reduce their toxicity. However, each anticancer drug has its own physicochemical, pharmacokinetic, and pharmacodynamics properties, which determine its unique clinical efficacy/safety. It is unknown if a universal nano delivery system using the same design principle can overcome the distinct shortcomings and improve the
unique efficacies of different anticancer drugs, especially when tumor EPR in human is questionable. In addition, it is unknown how do different nano carriers alter pharmacokinetics and mass balance of the drugs, which in turns affects drug’s efficacy/safety. Then, what should be selection criteria for different nano carriers based on the physicochemical, pharmacokinetic, and pharmacodynamics properties of the delivered drugs?

In this study, we investigated the three anticancer nanomedicine design principles and their clinical translations by evaluating the distinct clinical efficacy/safety of five clinically approved nanomedicines with different nano features (such as size, structure, composition, and long/short circulation), and comparing to the delivery efficiency in three types of preclinical cancer models. Specifically, we intend to answer the following question. Is nanomedicine accumulation by EPR in subcutaneous, orthotopic xenograft cancer and spontaneous transgenic cancer translated to clinical efficacy in cancer patients? Is the difference for the delivery efficiency/efficacy between preclinical xenograft cancers and human cancers consistent with tumor heterogeneity hypothesis in human? What are the mechanisms for the unique efficacy/safety profiles of each successful nanomedicines in cancer patients? Does long circulating nanomedicine design violate mass balance principle and how does it alter tissue distribution/penetration, which subsequently change efficacy/safety? Is it feasible to develop a universal nano delivery system with the same principle for different anticancer drugs that have distinct physico-chemical, pharmacokinetics, pharmacodynamics properties, and clinical efficacy/safety profiles? How do different nano carriers alter pharmacokinetics, mass balance, and tissue targeting of the delivered drugs that impacts efficacy/safety? What should be the new criteria to design anticancer nanomedicines to improve their clinical efficacy/safety in human cancer patients?
Results

Enhanced tumor accumulations of long circulating nanomedicines in xenograft model through EPR effect grossly exaggerated the delivery efficiency that has little clinical translation

To investigate if the nanomedicine design based on tumor EPR and long systemic circulation can be translated into observed clinical efficacy, the delivery efficiency of two clinically approved long circulating nanomedicines (PEGylated liposome Doxil® 85 nm, un-PEGylated liposome Myocet® 180 nm) in comparison with free doxorubicin were evaluated in three different animal cancer models. The delivery efficiency in these preclinical models were compared to their observed clinical efficacy in breast cancer patients.

Three different mouse cancer models (spontaneous transgenic breast cancer, orthotopic, subcutaneous xenograft breast cancer) with the same cancer type and same genetic background were used to minimize the variability. MMTV-PyMT transgenic spontaneous breast cancer mouse (FVB/NJ) was established by overexpression of large T antigen. The orthotopic mouse breast cancer by implanting PyMT breast cancer cells in the mammary fat pad of FVB/NJ mice. The subcutaneous xenograft breast cancer model was established by implanting the PyMT cancer cells subcutaneously in the flank of FVB/NJ mice.

In comparison with free doxorubicin, Doxil® (PEGylated liposome of doxorubicin, 85 nm) and Myocet® (non-PEGylated liposome of doxorubicin, 180 nm) maintained high plasma concentration where PEGylated liposomal Doxil® has higher plasma concentration than Non-PEGylated liposomal Myocet® (Fig 1A, D, G). These data clearly show the long systemic circulation of these two nanomedicines.

Long circulating Doxil® and Myocet® showed significant higher tumor accumulation (2 to 5-fold) in subcutaneous breast cancer (Fig 1B) and in orthotopic breast cancer (Fig 1E) compared to free
doxorubicin. The PEGylated liposomal Doxil® achieved 1.5 to 2-fold higher tumor accumulation than non-PEGylated liposomal Myocet®, which are consistent with previous studies for their tumor accumulations and superior efficacy in subcutaneous xenograft model 33-34. However, Doxil® and Myocet® did not increase drug tumor accumulation in transgenic spontaneous breast cancer that better mimic human breast cancer, rather they showed similar drug concentrations in tumors from 2-72 hrs compared to doxorubicin (Fig 1H). These data suggest that long circulating nanomedicines enhanced tumor accumulation only in subcutaneous and orthotopic cancer, but did not improve tumor accumulation through EPR effect in transgenic spontaneous PYMT breast cancer model, regardless particle size (80-180 nm) and composition. In contrast, free doxorubicin has similar tumor accumulations in three different cancer models (Fig 1 B, E, H).

However, the clinical studies showed that neither Doxil® nor Myocet® has superior efficacy in breast cancer patients in comparison with free doxorubicin, although these two nanomedicines showed significantly better efficacy in animal cancer models37-41. Indeed, tumor accumulation of Doxil® in human breast cancer patients did not provide direct evidence to support nanomedicine accumulations by EPR effect 35-36, 38-43 since the studies did not compare with free doxorubicin in humans. These data suggest that, unlike small molecules with similar tumor accumulation in three different cancer models, the enhanced tumor accumulation by EPR for the long circulating nanomedicines in the subcutaneous or orthotopic xenograft cancer model may grossly exaggerates the delivery efficiency and efficacy of nanomedicine. These data neither translated in the clinical efficacy in breast cancer patients, nor correlated to the drug accumulation in the transgenic spontaneous breast cancer.
Figure 1. PEGylated liposomal Doxil® and Non-PEGylated liposomal Myocet® nanomedicines did not enhance tumor drug accumulation in transgenic spontaneous breast cancer although they showed significant tumor drug accumulation in subcutaneous and orthotopic breast cancer through EPR effect. (A, B, C) Drug concentration in plasma, tumor, and tumor/plasma concentration ratio in subcutaneous breast cancer model after IV dose of doxorubicin, Doxil, and Myocet (5 mg/kg). (D, E, F) Drug concentration in plasma, tumor, and tumor/plasma concentration ratio in orthotopic breast cancer model after IV dose of doxorubicin, Doxil, and Myocet (5 mg/kg). (G, H, I) Drug concentration in plasma, tumor, and tumor/plasma concentration ratio in MMTV-PyMT spontaneous breast cancer model after IV dose of doxorubicin, Doxil, and Myocet (5 mg/kg).
**Long circulation nanomedicine design without adequate tumor EPR violates mass balance principle to improve clinical efficacy due to reduced tissue distribution/penetration in tumor**

One hypothesis is that long circulating nanomedicine design is not only to increase tumor drug accumulation by EPR, but also to enhance tumor/plasma ratio even without adequate EPR. However, long circulating nanomedicine design without adequate tumor EPR may violate mass balance principles to improve anticancer efficacy since it may reduce tissue distribution and limit anticancer efficacy.

Our data showed that long circulating nanomedicine decreases tumor/plasma ratio and tumor penetration. In comparison with doxorubicin, the long circulating Doxil® significantly decreased tumor/plasma ratio by 50 to 300-fold compared to doxorubicin regardless cancer models with/without adequate EPR (Fig 1C, F, I), while doxorubicin has similar highest tumor/plasma ratio in all three cancer models. Non-PEGylated Myocet® decreased tumor/plasma ratio by 25-fold (Fig 1C, F, I).

The tumor penetration of these nanomedicines was visualized by confocal imaging for doxorubicin-based drug nanomedicines in transgenic spontaneous breast cancer (Fig 2A-C). Doxil® (Fig 2B) and Myocet® (Fig 2C) have poor tumor tissue penetration and stay close to blood vessels as stained by anti-CD31 antibody, while doxorubicin can distribute throughout the tumor (Fig 2A). The penetration distance from the blood vessels was approximately 50% lower with Doxil and Myocet treated groups compared to the doxorubicin group (Fig 2D).

In 17 normal organs, long circulating Doxil® uniformly decrease tissue accumulation in most organs except skin, kidney, spleen, and lung in comparison with free doxorubicin (Fig S3). The tissue/plasma ratio from Doxil® is significantly low in all organs in comparison with doxorubicin (S4). Similarly, Myocet® has low tissue distribution in most organs except skin, liver, and spleen.
The tissue/plasma ratios of Myocet® in all organs are significantly lower than that of doxorubicin (Fig S3, S4). These data suggest that Doxil® and Myocet® have significantly lower tissue penetration compared to doxorubicin. Therefore, Doxil® and Myocet® were less likely to reach cancer cells, without adequate accumulation by EPR, to improve their anticancer efficacy in these organs due to the low tissue distribution/penetration.

Fig 2.

A DAPI (Nucleus)  CD31 (Blood vessels)  Doxorubicin  Overlay (CD31/Doxorubicin)

B

Doxil

C

Myocet

D

Drug penetration from tumor vasculature ratio (% vs Doxorubicin)

- Doxorubicin
- Doxil
- Myocet
Figure 2. Long circulating PEGylated Doxil and Non-PEGylated Myocet decreases tumor penetration as measured by confocal fluorescent imaging. (A) Doxorubicin distribution (Red) in PyMT breast tumor tissues, that is overlaid with blood vessels (green, anti-CD31 staining) and nuclear staining (Blue, DAPI staining) after IV dose (5 mg/kg). (B) Doxil distribution (Red) in PyMT breast tumor tissues, that is overlaid with blood vessels (green, anti-CD31 staining) and nuclear staining (Blue, DAPI staining) after IV dose (5 mg/kg). (C) Myocet distribution (Red) in PyMT breast tumor tissues, that is overlaid with blood vessels (green, anti-CD31 staining) and nuclear staining (Blue, DAPI staining) after IV dose (5 mg/kg). (D) Average distance of doxorubicin distributed away from blood vessels as measured by Imaging analysis from A-C.

The preferred skin accumulation of long circulating Doxil® and Myocet®, not tumor EPR, is correlated with their clinical efficacy in ARKS patients and caused hand-foot syndrome

Long circulating nanomedicines Doxil® and Myocet® showed superior efficacy to treat AIDS-related Kaposi’s sarcoma (ARKS) but not on other solid cancers, in comparison with free doxorubicin. It was hypothesized that ARKS (but not other solid cancers) has better EPR effect for nanomedicine accumulation in tumor tissues. However, it is not clear if superior efficacy of Doxil in human ARKS is due to high accumulation by EPR in the tumor or it is simply due to skin accumulation since no comparison with normal skin tissues was investigated. In addition, both Doxil® and Myocet® cause hand-foot syndrome. Our data showed Doxil® exclusively accumulated 4 to 6-fold higher in skin than doxorubicin (Fig 3A), which is correlated with its superior efficacy and its unique adverse reaction of hand-foot-syndrome. Surprisingly, skin is the only tissue that Doxil® was accumulated the highest along with few other tissues (Fig S3). Myocet® also had high level of accumulation in the skin but it is lower than that of Doxil®, which correlated similar superior efficacy in ARKS but lower incidence of hand-foot syndrome compared to Doxil®. These data suggest that both superior efficacy and hand-foot-syndrome of these two nanomedicines may be due to the preferred drug accumulation in the skin.
Decreased tissue distribution in the heart and mammary fat pad of Doxil® and Myocet® was associated with their reduced cardiotoxicity and lack of superior efficacy in breast cancer.

The most significant advantage for Doxil® and Myocet® is the reduced cardiomyopathy, which may due to their decreased tissue distribution/penetration in the heart. Our data showed that the accumulation and tissue/plasma ratios of Doxil® and Myocet® in the heart and all tissues are extremely low (Fig 3B), which explain why these two nanomedicines significantly reduce cardiotoxicity of doxorubicin. The accumulation in most other tissues including breast fat pad are significantly lower than that of free doxorubicin (Fig 3C, S3-S4), which may explain why Doxil® and Myocet® did not show superior efficacy to treat breast cancer in comparison to doxorubicin 20, 35-36, 38-43.

**Figure 3.** Tissue targeting of long circulating nanomedicines (Doxil and Myocet), not EPR in tumors, is associated with their anticancer efficacy and adverse reactions in comparison with free drugs. (A) Doxil achieved highest accumulation in skin, (B) lower accumulation in the heart, and (C) lower accumulation in the mammary fat pad compared to Myocet and doxorubicin (after IV dose 5 mg/kg), which is associated with its clinical efficacy and safety in comparison with doxorubicin. MMTV-PyMT mice were IV dosed with same dose of different nanoformulations. At different time points, three mice were sacrificed to collect blood and other normal organs. The drug concentrations in each tissues were measured using LC-MS/MS.
The tumor accumulation of short circulating nanomedicines in xenograft cancer and spontaneous transgenic cancer are inconsistent with observed clinical efficacy

To investigate if the tumor accumulation of short circulating of nanomedicines can be translated into observed clinical efficacy, we investigated the delivery efficiency of three clinically approved short circulating nanomedicines Abraxane®, Genexol-PM®, and Paclical® in three different animal cancer models in comparison with their free paclitaxel (Taxol® micelle). The delivery efficiency in preclinical model was compared to their observed clinical efficacy in breast cancer patients.

In comparison with paclitaxel (Taxol®), Abraxane® (albumin nanoparticle of paclitaxel, 130 nm), Genexol-PM® (PEG-PLA polymeric nanoparticle of paclitaxel, 22 nm), and Paccial® (all-trans retinoic acid micellar nanoformulation of paclitaxel, 42 nm) have a 3-5 fold lower plasma concentration-time profile (Fig 4A, D, G). These data suggest the short circulation time of these three nanomedicines in the blood in comparison with Taxol® (paclitaxel micelle formulation).

In comparison with paclitaxel (Taxol®), short circulating Abraxane®, Genexol-PM®, and Paccial® showed slightly higher (1.5 to 2-fold) tumor accumulation in subcutaneous xenograft breast cancer and in orthotopic xenograft breast cancer (Fig 4B, E). No significant differences were observed among three different nanomedicines regardless their size (22, 42, 136 nm) and composition (Albumin, PEG-PLA, all-tans retinoid acid) in these models. However, these nanomedicines (Abraxane®, Genexol-PM®, Paccial®) showed 50% lower tumor accumulation than paclitaxel (Taxol®) in clinically relevant transgenic spontaneous PYMT breast cancer model (Fig 4H). These data suggest that short circulating nanomedicines slightly enhanced tumor accumulation in subcutaneous and orthotopic xenograft cancer, but did not improve tumor accumulation in transgenic spontaneous PYMT breast cancer model, regardless particle size (20-130 nm),
composition, and structures. In contrast, Taxol (paclitaxel micelle) has similar tumor accumulations in all three different cancer models (**Fig 4B, E, H**).

Interestingly, clinical studies showed that these nanomedicines had very distinct efficacy/safety profiles, which are inconsistent with their tumor accumulation in different mouse cancer models. For instance, Abraxane has superior efficacy in treating metastatic breast cancer in comparison with free paclitaxel \(^{19, 46, 53-55}\), while Genexol-PM showed non-inferior (may be superior) efficacy in phase III clinical trials on breast cancer patients \(^{58}\) and Paclical was approved for ovarian cancer. These data suggest that, unlike small molecules with similar tumor accumulation in three different cancer models, the different preclinical cancer models have distinct ability for nanomedicine accumulation, which is inconsistent with their distinct clinical efficacies.
Figure 4. Nanomedicines of paclitaxel did not enhance tumor drug accumulation in transgenic spontaneous breast cancer although they showed significant tumor drug accumulation in subcutaneous and orthotopic breast cancer through EPR effect. (A, B, C) Drug concentration in plasma, tumor, and tumor/plasma concentration ratio in subcutaneous breast cancer model after IV dose of paclitaxel (Taxol®), Abraxane®, Genexol-PM®, and Paclical® (10 mg/kg). (D, E, F) Drug concentration in plasma, tumor, and tumor/plasma concentration ratio in orthotopic breast cancer model after IV dose of paclitaxel (Taxol®), Abraxane®, Genexol-PM®, and Paccimal® (10 mg/kg). (G, H, I) Drug concentration in plasma, tumor, and tumor/plasma concentration ratio in MMTV-PyMT spontaneous breast cancer model after IV dose of paclitaxel (Taxol®), Abraxane®, Genexol-PM®, and Paccimal® (10 mg/kg).
Short circulating nanomedicine design may increase tissue distribution/penetration to improve clinical anticancer efficacy

From mass balance perspective, short circulating nanomedicines in blood circulation may be due to either high tissue distribution or high elimination. High tissue distribution of nanomedicines likely increases anticancer efficacy in the target organ while high elimination is detrimental to their efficacy. Our data showed, in comparison with Taxol®, three short circulating nanomedicines (Abraxane®, Genexol-PM®, Paclical®) increased tumor/plasma ratio in subcutaneous and orthotopic xenograft cancer (Fig 4C, F), but did not alter the tumor/plasma ratios in spontaneous breast cancer (Fig 4I). The Mass spectrometry imaging of paclitaxel did not show any significant difference in tumor distribution/penetration in all three nanomedicines in comparison with Taxol® in transgenic spontaneous breast cancer (Fig 5).

In 17 normal organs, three short circulating nanomedicines of paclitaxel seem to improve tissue distribution/penetration in most of the organs (Fig 5S). More importantly, these three nanomedicines showed preferential penetration in several target organs in comparison with free paclitaxel (Fig S6), which may correlate with their clinical efficacy 65, 71 (see below).
Figure 5. Short circulating nanomedicines of paclitaxel did not alter tumor penetration as measured by Mass spectrometry imaging (MSI). (A) Paclitaxel (Taxol®), distribution (green and yellow) in PyMT breast tumor tissues after IV dose (50 mg/kg). (B) Abraxane® distribution (green and yellow) in PyMT breast tumor tissues after IV dose (50 mg/kg). (C) Genexol-PM® distribution (green and yellow) in PyMT breast tumor tissues after IV dose (50 mg/kg). (D) Paclical® distribution (green and yellow) in PyMT breast tumor tissues after IV dose (50 mg/kg). The MSI was overlaid with blood vessel staining (brown, anti-CD31 staining) Bar = 50 um. The frozen sections were prepared and coated with TiO2 matrix for MS imaging analysis.

The preferred tissue distribution/penetration in pancreas, lung and breast tissues of short circulating Abraxane® correlates with its superior efficacy in solid cancers of these organs

The decreased plasm concentration (3 to 5-fold) of short circulating Abraxane®, Genexol-PM® and Paclical® can not explain the their unique clinical efficacy in comparison with paclitaxel 19, 44-55.
However, our data showed that although Abraxane® has 3 to 5-fold lower plasma concentration vs. paclitaxel (Taxol®), it achieved similar concentrations to paclitaxel (Taxol®) in pancreas and lung (and slightly lower in breast) (Fig 6 A, B, C), while the tissue/plasma ratios of Abraxane® were significantly higher (2 to 4-fold) in pancreas, lung, and breast fat pad than paclitaxel (Fig 6A, B, C), which correlated with Abraxane®’s superior efficacy to treat these three types of cancers in comparison with paclitaxel (Taxol®) 19,47,50,65. In comparison, Genexol-PM® showed non-inferior efficacy (may be superior) to paclitaxel in a phase III clinical trials to treat breast cancer 58,72. Our data indeed showed that Genexol-PM® improved tissue/plasma ratios in breast, which explain the clinical data. However, Genexol-PM did not increase tissue penetration in the pancreas and lung, which may suggest this nanomedicine may not have efficacy in pancreatic or lung cancer.

It is worth noting that clearance of Abraxane® and paclitaxel in human is slower than mice 73, which may further enhance tissue distribution/penetration in human for its superior efficacy. By comparing pharmacokinetics of Abraxane® and Paclitaxel in both human 63-64, 66 and mouse, we fund that the mouse cleared paclitaxel from blood 3 to 5-fold faster than human based on plasma pharmacokinetics, which was also reported in previous studies 73. Our previous studies showed that more than 40-50% of Paclitaxel and Abraxane® were cleared from blood within 5-10 min after IV administration in mice 71,74. Despite the fast clearance in mice, Abraxane® showed preference in tissue distribution/penetration than paclitaxel. To estimate drug distribution/penetration of Abraxane® and paclitaxel in human tissues, we performed pharmacokinetic simulation of Abraxane® and paclitaxel as we described in previous study 75 (Fig 6D). The simulation showed that Abraxane® distribution/penetration in human tissue (as shown by tissue /plasma AUC ratio) is 1200-fold, which is significantly higher than paclitaxel’s tissue distribution/penetration (300-fold). In contrast, Abraxane® distribution/penetration in mouse tissue (as shown by tissue /plasma
AUC ratio) is 3-fold, which is also significantly higher than paclitaxel’s tissue distribution/penetration (0.5 to 1-fold). These data suggest that Abraxane® has significant higher tissue distribution/penetration in human tissues than paclitaxel, which is correlated with its superior efficacy in treatment of pancreatic, lung and breast cancers.

Fig 6.
Figure 6. Tissue targeting of short circulating nanomedicines (Abraxane, Genexol-PM, Paclical), not EPR in tumors, is associated with their anticancer efficacy in comparison with free drugs. (A) Abraxane® achieved higher tissue/plasma ratios in pancreas (A), lung (B), and (C) breast fat pad than Paclitaxel (TAXOL®), which is associated with its superior efficacy in comparison with paclitaxel. (D) The tissue distribution of Abraxane and paclitaxel in mouse by pharmacokinetic simulation in mouse after IV of 10 mg/kg and in human after 175-260 mg/m2 dose. MMTV-PyMT mice were IV dosed with same dose of different nanoformulations. At different time points, three mice were sacrificed to collect blood and other normal organs. The drug concentrations in each tissues were measured using LC-MS/MS. Human were dosed with 175-260 mg/m2 paclitaxel or Abraxane by IV infusion (30 min–3hrs).

The decreased blood concentration of Abraxane® was correlated with the reduced neutropenia, while the higher gastrointestinal tract secretion increased GI toxicity.

Short-circulating Abraxane® has 3 to 5-fold lower plasma concentration vs. paclitaxel (Taxol®) (Fig 2). Our previous study using mouse albumin nanoparticle of paclitaxel showed that it reduced blood/plasm AUC ratio, which suggest low blood cell uptake. These data explains why Abraxane® has lower incidence of neutropenia in comparison with paclitaxel. In addition, our previous study showed that Abraxane® enhanced secretion to gastrointestinal track in comparison with paclitaxel, which was correlated with the increased GI toxicity of Abraxane® in comparison to paclitaxel.

Less than 1% of nanomedicines were delivered to tumors, regardless nano size, structure, composition, long/short-circulation, anticancer efficacy, or tumor models.

Achieving a high percentage of injected dose delivered to xenograft tumor is often used as a criterion for nanomedicine design. However, most nanomedicines are only able to achieve low percentages of injected dose in xenograft tumors by previous reports. Four critiques were raised for the previous reports. (1) are the calculation methods (based on AUC) and data from literature accurate for percentage of dose injected in the tumors? (2) are the data from different nano delivery systems with different materials relevant to human? (3) what is the clinical relevant percentage of drug delivered to tumor for clinical efficacy? (4) what is percentage injected dose of small molecule itself? To answer these critiques, we measured the percentage of dose delivered...
into tumors in two different cancer models by measuring drugs in all tissues and tumors at six different time points.

Our data showed that the percentages of injected dose in the tumor are in the range of 0.2% - 1% for all five evaluated nanomedicines in all three cancer models, which is independent of nano size, structure, composition, long/short-circulation, anticancer efficacy, or tumor models (Fig 7). In comparison, the percentage of injected dose of small molecules in the tumors were in the range of 0.2-0.6%. The percentage of dose of nanomedicines and small molecules in the tumor was variable at different time points (Fig 7).

In the subcutaneous and orthotopic xenograft breast cancer model with significantly enhanced tumor accumulations (2 to 5-fold) by EPR effect for long circulating nanomedicines, there were no difference in the percentage of dose injected in the tumor from 5 min – 7 hrs among Doxil®, Myocet®, and free doxorubicin. At later time points (24-72 hrs), the percentage of Doxil® in the tumors (0.6%-1%) was higher than that of Myocet® and doxorubicin (<0.2%-0.4%) (Fig 7A, 7B). However, despite the low percentage of drug delivered into the xenograft tumors, previous studies have repeatedly showed significant advantage in anticancer efficacy of Doxil® and Myocet® in xenograft model. Again, these differences were not observed in the transgenic breast cancer model from 5 min to 72 hrs, where 0.3-0.5% of injected dose was delivered into the tumors (Fig 7C).

Similarly, in the subcutaneous xenograft breast cancer model with reasonable enhanced tumor accumulations (2-fold) by EPR, the short circulating paclitaxel-based nanomedicines showed no difference in the percentage of injected dose in tumors at early time points (5 min to 4 hrs), but slightly higher (0.1%) than free paclitaxel (0.03%) at later time point (24 hrs) (Fig 7D). The percentage of nanomedicines in orthotopic cancer were slightly higher than that of free paclitaxel (Fig 7E), but there were no difference in the transgenic breast cancer model from 5 min to 24 hrs.
(Fig 7F). However, preclinical and clinical studies already showed these nanomedicines have superior efficacy than free drugs. These data suggest that achieving a high percentage of injected dose in tumor may not be a realistic or necessary criterion for preclinical or clinical efficacy. The lower percentage of nanomedicines and small molecules in tumors (<0.2% - 1%) is adequate for anticancer efficacy. The advantage of nanomedicines for improve anticancer efficacy may be due to tissue targeting rather than tumor accumulation as discussed above.

Fig 7

Subcutaneous xenograft cancer

**A**

Doxorubicin

**D**

**B**

**E**

Orthotopic breast cancer

**C**

**F**

Spontaneous transgenic PyMT cancer
Fig 7. Percentage of dose injected in tumors in subcutaneous, orthotopic xenograft breast cancer, and spontaneous transgenic PyMT breast cancer models. Percentage of dose injected of long circulating nanomedicines (Doxil and Myocet) vs. free drug (doxorubicin) (10 mg/kg) in the subcutaneous xenograft breast cancer (A), orthotopic breast cancer (B), and spontaneous transgenic PyMT breast cancer model (C). Percentage of dose injected of short circulating nanomedicines (Abraxane, Genexol-PM and Paclical) vs. paclitaxel (Taxol) (10 mg/kg) in the subcutaneous xenograft breast cancer (D), orthotopic breast cancer (E), and spontaneous transgenic PyMT breast cancer model (F). The mice were IV dosed with same dose of different nanoformulations. At different time points, three mice were sacrificed to collect blood and other normal organs. The drug concentrations in tumors were measured using LC-MS/MS.

**Percentage of injected dose in the liver and spleen may not be a major concern for clinical efficacy/safety of nanomedicines based on the properties of the delivered drugs**

High percentage of injected dose in the liver and spleen is always a concern for short circulating nanomedicines due to the rapid clearance of RES system in the liver and spleen. However, it is not clear if high levels of nanomedicines in the liver and spleen is detrimental for their clinical efficacy/safety. Compared to doxorubicin, PEGylated liposomal Doxil® indeed reduced accumulation by liver the spleen while non-PEGylated liposomal Myocet increased accumulation in the liver and the spleen (Fig 8A, C). In addition, more than 10-15% of injected dose of both nanomedicines and free doxorubicin was accumulated in the liver, which is the highest among all tissues (Fig S1, S3, S4). However, clinical studies showed no difference for the toxicity of these two nanomedicines and free doxorubicin in the liver and spleen. The reduced accumulation in the liver and spleen of PEGylated liposomal Doxil® indeed increased tumor accumulation in xenograft breast cancer (but not in transgenic spontaneous breast cancer) in comparison with non-PEGylated liposomal Myocet®. However, clinical studies showed both nanomedicines have similar superior efficacy in treating ARKS, but no efficacy difference in treating breast cancer in comparison with free doxorubicin.

Similarly, compared to free paclitaxel, both free drug and nanomedicines (Abraxane®, Genexol-PM®, Paclical®) had high accumulations in the liver (Fig 8C), intestine, bone, skin, and muscle.
Fig S2, S5), but lower in the spleen (Fig 8D). In the liver, there are no differences for drug accumulations (>20%) among free paclitaxel and three nanomedicines regardless their size, composition, structures. In the spleen, free paclitaxel has higher drug accumulation than three nanomedicines. However, there is no reported dose-limiting toxicity differences in the liver and spleen for these three nanomedicines in comparison with free drugs. These data suggest that drug accumulations in the liver or spleen may not be useful criteria for clinical efficacy and safety unless liver and spleen toxicity are concern for the free drugs.

**Fig 8**

![Graphs showing drug accumulation in liver and spleen](image)

Fig 8. The levels of long and short circulating nanomedicines in the liver and spleen. Non-PEGylated liposome Myocet® has higher accumulation than PEGylated liposome Doxil® and free doxorubicin in (A) the liver and (B) the spleen. Similar levels of nanomedicines (Abraxane®, Genexol-PM®, Paclical®) and paclitaxel in (C) the liver, but lower levels of nanomedicines than paclitaxel in the spleen (D). Same dose of nanomedicines and free drugs were dosed in the spontaneous transgenic PyMT breast cancer mice. The tissues were collected at different time and drug concentrations were measured using LC-MS.
Discussions

**Nanomedicine design based on tumor EPR is only valid in mouse xenograft cancer, which exaggerates delivery efficiency/efficacy with little clinical translation in human patients.**

Clearly, there is discrepancy for the delivery efficiency and anticancer efficacy of nanomedicines between preclinical cancer in animal and in human cancer patients, which is also inconsistent with EPR heterogeneity hypothesis. The heated debate for tumor EPR may have focused on two very different questions: does EPR or EPR heterogeneity exist in mouse or human tumors? or can tumor EPR enhance nanomedicine accumulation to improve anticancer efficacy in mouse or human tumors? As a physiological phenomenon, the EPR or heterogeneous EPR heterogeneity may exist in different types of cancer, different stages of cancer in both mouse and human if a dye-labeled protein molecules (<5 nm) was used to assess EPR as it was first discovered. Clearly, EPR in subcutaneous or orthotopic xenograft cancers can enhance nanomedicines accumulation to improve anticancer efficacy. However, there is no direct evidence to support that EPR in human cancers (or transgenic mouse cancers) can enhance nanomedicine tumor accumulation, regardless of its heterogeneity, since nanoparticles are usually much larger (30-200 nm) than dye-labeled protein (<5 nm). Clinical studies to support the enhanced tumor accumulation of nanomedicine lacks either free drug controls or normal tissue controls. Our data and literature did not support the nanomedicine design principle based on tumor EPR to improve clinical anticancer efficacy in human cancer patients.

Consequently, our data also raised the questions for the validity of subcutaneous or orthotopic xenograft cancer models for evaluation of delivery efficiency and efficacy of most nanomaterials, which may exaggerate the delivery efficiency/efficacy but has little clinical translations. Unlike small molecules that are distributed similarly into different tumors (subcutaneous, orthotopic,
spontaneous cancer), nanomedicines are artificially enhanced tumor accumulation by the “intrinsic properties” of xenograft tumors, which may be “an artifact” of the model and grossly exaggerates the results. In reality, the clinical translation for the small molecules from preclinical xenograft models to human has been challenging due to different biology between animals and humans. The artificially exaggerated tumor accumulation of nanomedicines make the translation even worse. In such case, the spontaneous cancer model is critical to evaluate the delivery efficiency of the nanomedicine in the tumors of the targeted organs. Furthermore, the primary focus for treatment of clinical cancer patients is to treat metastatic disease since the primary tumors are often surgically removed. However, most nanomedicines are mainly tested for the delivery efficiency and efficacy against primary xenograft tumor with exaggerated outcome, but not on metastatic disease in preclinical models, which may have little clinical relevance.

Nanomedicine design based on long systemic circulation without adequate tumor EPR violates mass balance principles for improving efficacy by reducing tissue distribution/penetration

Without enhanced tumor accumulation by EPR in human cancer, long circulation nanomedicine design is self-conflicting strategy since it violates mass balance principles for improving anticancer efficacy by reducing tissue distribution/penetration. To achieve high plasma concentration for nanomedicines is contradicting with mass balance principle for better tissue distribution/penetration, which should not be used as a nanomedicine design criteria. This raised question for the PEGylation of most nanoparticle delivery systems with purpose to increase anticancer efficacy. The similarity of efficacy PEGylated liposome Doxil and Non-PEGylated liposome Myocet questioned the universal requirement for PEGylation of nanoparticles although in many other cases that PEGylations are required. In contrast, the high levels of accumulation
in the spleen and liver of Myocet did not affect its efficacy and toxicity, but reduced hand-foot-syndrome\textsuperscript{70}.

**It is not feasible to develop a universal nano delivery system based on the same design principle for different anticancer drugs that have distinct properties**

Different anticancer drugs have their distinct physico-chemical, pharmacokinetic, and pharmacodynamics properties, which determine their unique clinical efficacy and adverse events in human cancer patients. If the nanomedicine design based on EPR and long circulation is invalid in human cancer patients, then a universal nano delivery system is unlikely to overcome the distinct shortcomings, and improve their unique anticancer efficacy of different anticancer drugs. For instance, PEGylated liposome (Doxil) can overcome doxorubicin’s cardiotoxicity by reducing the tissue distribution/penetration in heart\textsuperscript{35-43, 77}, but it may not be suitable to deliver paclitaxel since paclitaxel’s limitation is poor tissue penetration. Rather, PEGylated liposome of paclitaxel may further reduce the tissue penetration to reduce its efficacy. In contrast, albumin nanoparticle (Abraxane) can overcome paclitaxel’s poor tissue distribution/penetration to improve paclitaxel efficacy in pancreatic, lung and breast cancer\textsuperscript{19, 58}, but it may not be suitable to deliver doxorubicin since albumin nanoparticle may not reduce drug accumulation in heart to overcome cardiotoxicity of doxorubicin, while tissue penetration of doxorubicin is not its limitation.

**Drug-specific nano delivery system need to be developed to overcome the intrinsic shortcomings of the delivered drugs to improve their clinical efficacy/safety profiles.**

(1). **Identify the intrinsic shortcomings of the delivered drugs on their physico-chemical, pharmacokinetic, and pharmacodynamic properties, which may be overcame by nanomedicines.**

Physico-chemical, pharmacokinetic, pharmacodynamic properties of the drugs demand different nano delivery systems. For instance, physico-chemical properties of doxorubicin (salt form) do
not have significant shortcomings, which is easily formulated to an injection formulation. The pharmacodynamic property of doxorubicin determines its wide spectrum of anticancer efficacy in various types of cancers. The pharmacokinetic properties of doxorubicin include extremely low plasma concentration and significant high tissue distribution/penetration after IV administration as shown by its rapid distribution from blood to all tissues (Fig S3), which reached equilibrium in all tissues as measured by tissue/plasma ratios (Fig S4). The high tissue distribution/penetration contributes its wide spectrum anticancer efficacy. However, the high distribution/penetration of doxorubicin in heart is the major limitation to cause cardiomyopathy. Therefore, the nanomedicine design (such as PEGylated liposome Doxil®) to reduce tissue distribution/penetration in heart tissues is desired to reduce cardiotoxicity. But Doxil® also decreases tissue distribution/penetration to all other organs (except skin), which explains why Doxil® does not improve anticancer efficacy in breast cancers compared to conventional doxorubicin.

In contrast, the physico-chemical property of paclitaxel showed poor solubility, which is very difficult for formulation where Taxol used Cremophor EL as solubilizing agents that causes severe allergic reaction. The pharmacodynamics properties of paclitaxel shows neutropenia and neuropathy as adverse events (AE). The pharmacokinetic property of paclitaxel showed high plasma concentration that causes neutropenia, and low tissue distribution/penetration to target organs that may limit its efficacy in some type of cancer (such as pancreatic cancer). As shown in Figure S5-S6, when paclitaxel was I.V. administered, its plasma concentration is high while tissue concentration is lower or similar to plasma (Fig S5). Paclitaxel’s tissue distribution is limited and never reach equilibrium in all tissues as measured by tissue/plasma ratios (Fig S6). This suggests that the tissue distribution/penetration is a limitation for paclitaxel for its efficacy.
Therefore, nanomedicine design is desired to reduce its plasma concentration (to reduce neutropenia) and enhance tissue distribution/penetration to enhance its anticancer efficacy as seen in Abraxane® 19, 47, 50, 65, 71, 74.

(2) Understand the pharmacokinetics and tissue targeting of nano carriers that can alter mass balance of the drugs in the targeted organ with residual tumors to improve clinical efficacy/safety. Not only do the nano carriers show distinct pharmacokinetics, but they also alter the pharmacokinetics and mass balance of the delivered drug in the targeted organs, which will subsequently affect drug’s efficacy/safety in the targeted tissues (organs)74. If the nanomedicines do not accumulate in the target organ of interest, it will unlikely to reach cancer cells to show efficacy in the organ of interest with residual cancer or metastatic lesions. Clinical efficacy of currently approved nanomedicines of doxorubicin and paclitaxel suggest organ targeting determines nanomedicine’s efficacy and safety. Doxil®’s tissue targeting, not tumor EPR, is responsible for the three unique features: superior efficacy to treat ARKS (due to the increased skin accumulation) but not in breast cancer and ovarian cancer 20-21, 30-35, 37-41; increased hand-foot syndrome (due to the increased skin accumulation) 36, 42-43; and reduced cardiotoxicity (due to the reduced heart accumulation) 20, 35-36, 38-43, 68. The clinical study showed human ARKS lesion has higher levels of Doxil than doxorubicin may simply due to skin accumulation rather than EPR in tumor since no comparison with normal skin tissues was investigated 35.

In contrast, tissue targeting of short circulating Abraxane® also correlated with four unique features: superior efficacy to paclitaxel (TAXOL®) in treatment of pancreatic cancer, non-small cell lung cancer, and breast cancer (due to the increased distribution/penetration in these tissues) 19, 44-52, 65; reduced neutropenia19 (due to the reduced blood levels and blood cell uptake), and
increased neuropathy (may be due to the increased exposure to nerve cells) \(^{56-57}\), and increased GI toxicity (due to the increased GI tract secretion) \(^{19, 56-57}\).

(3) Select a long or short circulating nano delivery system based on the properties of the delivered drugs and purpose to improve efficacy or safety.

Long circulation nanomedicine design and high plasma concentration should not be universal criteria to improve clinical efficacy/safety. However, if the primary goal is to reduce drug toxicity (such as cardiotoxicity of doxorubicin), long systemic circulation nanomedicine design (Doxil®) is beneficial to reduce tissue distribution/penetration in the heart to reduce cardiotoxicity \(^{35-43}\). In contrast, if the primary goal is to improve drug’s efficacy, long systemic circulation nanomedicine design under inadequate EPR in human tumor may not be a valid strategy. Rather, short circulating nanomedicines (Abraxane® and Genexol-PM®) may enhance tissue distribution/penetration to improve efficacy in human \(^{19, 58}\).

**Conclusions**

Our data showed that nanomedicines enhanced tumor accumulation (3 to 5-fold) by EPR in subcutaneous and orthotopic xenograft cancer, but did not improve or even decreased tumor accumulation in spontaneous transgenic cancer model regardless nano size, structure, composition, and long/short circulation time. The delivery efficiency to tumors of different nanomedicines in preclinical cancer models did not correlate with observed clinical efficacy. The distinct efficacies/safeties of nanomedicines were determined by their distinct targeting properties to different tissues of the intended organs with residual tumors. In comparison with drug doxorubicin, the long circulating nanomedicines (Doxil®, Myocet®) preferentially target to skin, which were correlated their its superior clinical efficacy in AIDS-related Kaposi’s sarcoma and increased hand-foot syndrome in human patients; while they reduces tissue penetrations in all other organs.
(including heart) contributed to the reduced cardiotoxicity and lack of superiority in treating other solid cancers. In contrast, in comparison with paclitaxel, short circulating nanomedicine (Abraxane®) increased tissue distribution/penetration in the pancreas, lung, and breast for its superior clinical efficacy in pancreatic, lung and breast cancers in human patients. The Abraxane® decreased blood concentration to reduce incidence of neutropenia, but increased secretion to gastrointestinal tract to increase GI toxicity in human patients. Similarly, short circulating Genexol-PM increased tissue penetration in the breast for non-inferior (or superior) efficacy in human breast cancer, but failed to increase tissue penetration in the lung and pancreas that may suggest lack of efficacy for human pancreatic and lung cancer. Genexol-PM increased blood cell uptake that induces high incidence of neutropenia in human.

In addition, long systemic circulation of nanomedicine design, without adequate tumor accumulation by EPR, violates mass balance principles for improving efficacy by reducing drug tissue distribution/penetration. In contrast, short circulation of nanomedicine design may increase tissue distribution/penetration in the target organ to improve anticancer efficacy.

Our data suggest that nanomedicine design principles based on tumor EPR and long circulation are valid only in xenograft models, which exaggerate the delivery efficiency/efficacy with little clinical translation. It is not feasible to develop a universal nanoformulation based on the same design principle for different anticancer drugs with distinct properties. Rather, a drug-specific nanoformulation needs to be developed to overcome the intrinsic shortcomings of the delivered drug to improve clinical efficacy/safety in cancer patients, using three new criteria based on the drug’ distinct physico-chemical, pharmacokinetic, pharmacodynamic properties.
Materials and Methods

Materials

Taxol (paclitaxel injection, micelle formulation using Cremophor EL) was purchased from the Hospital Pharmacy of University of Michigan (Ann Arbor, MI). Abraxane (albumin-bound nanoparticle of paclitaxel) was provided by Celgene Corporation (Summit, NJ, U.S.A.). Paclical (The micellar formulation of paclitaxel encapsulated in the proprietary retinoid compound XR-17) was procured from the Russian Federation courtesy of Celgene Corporation, and Genexol-PM (polymer formulation of paclitaxel) was procured from the South Korea courtesy of Celgene Corporation. Doxorubicin hydrochloride solution and Doxil (PEGylated liposome from Janssen Oncology, Raritan, NJ, U.S.A.) were purchased from the Hospital Pharmacy of the University of Michigan. Myocet (Non-PEGylated liposomes of paclitaxel, GP-Pharm, Spain) was procured from the EU courtesy of Celgene Corporation.

Three types of breast cancer models

All animal procedures used in this study were approved by the University Committee on Use and Care Animals at the University of Michigan. We developed three types of breast cancer models, which have same genetic background and cancer cells to minimize variability. MMTV-PyMT transgenic spontaneous breast cancer model: The female MMTV-PyMT mice (FVB/NJ background) used in this study were 8-12 weeks old with tumor sizes of 150-500 mm$^3$. The breeding colonies were established by crossing FVB/NJ females (Stock No. 001800) with hemizygous FVB/N-Tg (MMTV-PyMT) 634Mul/J males (Stock No: 002374) from The Jackson Laboratory (Bar Harbor, ME, USA). Orthotopic breast cancer model and subcutaneous breast cancer models: PyMT breast cancer cells ($1 \times 10^6$ cells/0.1 mL/mouse) isolated from MMTV-PyMT mouse tumors suspended in a Matrigel/RPMI-1640 (50/50) mixture were inoculated into the
FVB/NJ female mice through subcutaneous (left inguinal fold) or orthotopic injection (fourth mammary fat pad).

The mice were injected through tail vein with four formulations of paclitaxel (Taxol-injectable paclitaxel with Cremophor EL, Abraxane, Genexol-PM, Paclical, 10 mg/kg), or three formulations of doxorubicin (Doxorubicin- injectable clinical used solution, Doxil, Myocet, 5 mg/kg) once average tumor sizes reached ~150 mm$^3$. Mouse tumor size were monitored and calculated based on the following formula: volume = $\frac{1}{2} \times$ length $\times$ width $\times$ width. Serial samples of blood, plasma, tumor, brain, fat, heart, intestine, kidney, liver, lung, muscle, pancreas, spleen, stomach were collected from pre-dose, 0.083, 1, 4, 7, 24, and 72 h (doxorubicin formulations only) post dose for drug concentration analysis, imaging analysis, and immunostaining, 3 mice/time point/group.

**LC-MS/MS analysis of drug concentration in plasma, blood, tumor, and other tissues**

The LC-MS/MS analysis of paclitaxel was performed using docetaxel as internal standard (IS). LC-MS/MS analysis of doxorubicin was performed using daunorubicin as internal standard (IS).

The analytical assay was validated according FDA guidance for linearity (2 to 5000 ng/mL), matrix effect, recovery, low detection limit, Quality control (QC) in different biological matrix, including plasma, blood, tumor, and each different organ homogenates. LC-MS/MS was performed using ABI-5500 Qtrap (Sciex, Ontario, Canada) mass spectrometer with electrospray ionization source was interfaced with Shimadzu high performance liquid chromatography (HPLC) system with Xbridge C18 column (50 $\times$ 2.1 mm ID, 3.5 $\mu$m). The mass spectrometer was operated in a positive mode with multiple reaction monitoring (MRM) for analysis. The MRM transitions were monitored at m/z 854.4 to 286.1 for paclitaxel and m/z 808.0 to 226.0 for internal standard. The MRM transitions were monitored at m/z 544.1 to 397.2 for doxorubicin and m/z 528.5 to 321 for internal standard.
Pharmacokinetic and Mass Balance Analysis

The Pharmacokinetic (PK) analysis from all formulations were compiled and calculated with Phoenix/WinNonlin (version 6.4; Pharsight, Mountain View, CA, U.S.A.) The plasma/blood and tissue concentration-time data were compiled. Efficiency of paclitaxel and doxorubicin delivery by different formulations was evaluated by comparing concentrations of paclitaxel or doxorubicin in plasma and tissues at each time point. The amounts of paclitaxel or doxorubicin were calculated as the products of the corresponding concentrations and the blood volumes or tissue weights. The percentage of injected dose in each tissue was calculated using the amount of paclitaxel or doxorubicin in each tissue per injected dose. The pharmacokinetic modeling and simulation were conducted using nonlinear mixed-effects modeling program (NONMEM, version 7.2; ICON Development Solutions, MD) with first-order conditional estimation using the INTERACTION (FOCEI) option 75. The tissue concentration and AUC were predicted in human and mouse based on plasma drug concentration as previously described 75.

Mass spectrometry imaging to visualize nanomedicine distribution in tumor tissues

Cryosectioned tumors of 10 µm thickness were mounted onto precooled glass plates or metal imaging plates. Then the slice were sprayed with TiO2 matrix suspension including 200 ng/mL reference compound D5-paclitaxel. MS is performed under negative mode to form in source fragment ions at M/Z 284.098 and M/Z 289.127, respectively. Cryosectioned tumors were imaged at a spatial resolution of 50 µm in negative ion mobility mode. A MALDI-ion mobility mass spectrometer Synapt G2-Si Qtof (Waters Corporations, USA) was used for imaging of the tissue samples. It is equipped with a 355 nm Nd:YAC laser with a 100-2500 Hz repetition rate. MS spectra were acquired with an automatic scan rate under sensitivity mode with positive or negative ionization method. The MALDI source settings was set with 0.5 scans per pixel, 1000 or 2000 HZ.
laser firing rate and 400 laser energy. HDImaging software V1.4 from Waters was used to process and display ions distribution inside the tissue sections.

**IHC staining of blood vessels in tumor tissues**

IHC staining of blood vessels in frozen section of primary tumor slides was performed by IVAC facility at the University of Michigan. Briefly, the cryosectioned tumor samples were incubated with anti-CD31 antibody (Dianova, Cat# DIA310, clone SZ31) after post-fixation in pre-chilled acetone at -20ºC for 10 min. The detection was performed by a horseradish peroxidase biotin-free polymer based commercial detection system, disclosure with dianinobenzidine (DAB) chromogen, and nuclear counterstaining with hematoxylin. Slides were scanned on Aperio At-2.

**Immunostaining and confocal imaging of blood vessels in tumor tissues**

Cryosectioned tumors of 10 µm thickness were mounted onto precooled glass plates. Immunostaining of blood vessels in frozen section of primary tumor slides was performed with anti-CD31 antibody (Abcam, Cat# ab28364) after post-fixation in IHC Zinc fixative (BD Pharmingen™) for 45 second. The detection was performed by Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488) (Abcam, Cat# ab150077). All immunostained slides were mount in ProLong™ Diamond Antifade Mountant with DAPI (Molecular Probes™, P36971) and imaged with Leica SP8 inverted MP confocal.

**Statistical Analysis.**

Statistical analysis was conducted using GraphPad Prism 7.0 software. One-way ANOVA was used if the comparison involved more than two groups. Two-tailed Student's t-test was used to compare the statistical difference between two groups. A P-value < 0.05 was considered significant.
Supplementary Materials

Fig. S1

Figure S1. Percentage of injected dose in 17 organs after IV dose doxorubicin, Doxil, Myocet from 0.083-72 hrs in MMTV-PyMT transgenic spontaneous breast cancer model. MMTV-PyMT mice were IV dosed with same dose of different nanoformulations of doxorubicin (5 mg/kg). At different time points, three mice were sacrificed to collect blood, tumor and other normal organs. The drug concentrations in each tissues were measured using LC-MS/MS. The amounts of doxorubicin were calculated as the products of the corresponding concentrations and the blood volumes or tissue weights. The percentage of injected dose in each tissue was calculated using the amount of paclitaxel in each tissue per dose.
Figure S2. Percentage of injected dose in 17 organs after IV dose paclitaxel, Abraxane, Genexol-PM, and Paclical from 1-24 hrs in MMTV-PyMT transgenic spontaneous breast cancer model. MMTY-PyMT mice were IV dosed with same dose of different nanoformulations of paclitaxel (10 mg/kg). At different time points, three mice were sacrificed to collect blood, tumor and other normal organs. The drug concentrations in each tissues were measured using LC-MS/MS. The amounts of paclitaxel were calculated as the products of the corresponding concentrations and the blood volumes or tissue weights. The percentage of injected dose in each tissue was calculated using the amount of paclitaxel in each tissue per dose.
Fig S3

Blood

- Doxorubicin
- Doxil
- Myocet

Bone

- Doxorubicin
- Doxil
- Myocet

Brain

- Doxorubicin
- Doxil
- Myocet

Fat

- Doxorubicin
- Doxil
- Myocet

Fat Pad

- Doxorubicin
- Doxil
- Myocet

Heart

- Doxorubicin
- Doxil
- Myocet

Intestine

- Doxorubicin
- Doxil
- Myocet

Kidney

- Doxorubicin
- Doxil
- Myocet

Liver

- Doxorubicin
- Doxil
- Myocet

Lung

- Doxorubicin
- Doxil
- Myocet

Muscle

- Doxorubicin
- Doxil
- Myocet

Pancreas

- Doxorubicin
- Doxil
- Myocet

Plasma

- Doxorubicin
- Doxil
- Myocet

Skin

- Doxorubicin
- Doxil
- Myocet

Spleen

- Doxorubicin
- Doxil
- Myocet

Stomach

- Doxorubicin
- Doxil
- Myocet

Tumor

- Doxorubicin
- Doxil
- Myocet

Uterus

- Doxorubicin
- Doxil
- Myocet
**Fig S4**

**Blood vs Plasma**

- Doxorubicin
- Doxil
- Myocet

**Bone vs Plasma**

- Doxorubicin
- Doxil
- Myocet

**Brain vs Plasma**

- Doxorubicin
- Doxil
- Myocet

**Fat vs Plasma**

- Doxorubicin
- Doxil
- Myocet

**Fat Pad vs Plasma**

- Doxorubicin
- Doxil
- Myocet

**Heart vs Plasma**

- Doxorubicin
- Doxil
- Myocet

**Intestine vs Plasma**

- Doxorubicin
- Doxil
- Myocet

**Kidney vs Plasma**

- Doxorubicin
- Doxil
- Myocet

**Liver vs Plasma**

- Doxorubicin
- Doxil
- Myocet

**Lung vs Plasma**

- Doxorubicin
- Doxil
- Myocet

**Muscle vs Plasma**

- Doxorubicin
- Doxil
- Myocet

**Pancreas vs Plasma**

- Doxorubicin
- Doxil
- Myocet

**Plasma vs Plasma**

- Doxorubicin
- Doxil
- Myocet

**Skin vs Plasma**

- Doxorubicin
- Doxil
- Myocet

**Spleen vs Plasma**

- Doxorubicin
- Doxil
- Myocet

**Stomach vs Plasma**

- Doxorubicin
- Doxil
- Myocet

**Tumor vs Plasma**

- Doxorubicin
- Doxil
- Myocet

**Uterus vs Plasma**

- Doxorubicin
- Doxil
- Myocet
Figure S3-S4. The concentrations and tissue/plasma concentration ratios of doxorubicin, PEGylated liposomal Doxil, and Non-PEGylated liposomal Myocet nanomedicines in 18 different tissues of MMTV-PyMT transgenic spontaneous breast cancer mice after IV dose of doxorubicin, Doxil, and Myocet (5 mg/kg). MMTY-PyMT mice were IV dosed with same dose of different nanoformulations of doxorubicin (5 mg/kg). At different time points, three mice were sacrificed to collect blood, tumor, and other normal organs. The drug concentrations in each tissues were measured using LC-MS/MS.
Fig S6

Blood vs Plasma

Bone vs Plasma

Brain vs Plasma

Fat vs Plasma

Fat Pad vs Plasma

Heart vs Plasma

Intestine vs Plasma

Kidney vs Plasma

Liver vs Plasma

Lung vs Plasma

Muscle vs Plasma

Pancreas vs Plasma

Plasma vs Plasma

Skin vs Plasma

Spleen vs Plasma

Stomach vs Plasma

Tumor vs Plasma

Uterus vs Plasma
Figure S5-S6. The concentrations and tissue/plasma concentration ratios of Taxol, Abraxane, Genexol-PM, Paclical in 17 tissues of MMTV-PyMT spontaneous breast cancer model after IV dose of Taxol, Abraxane, Genexol-PM, and Paclical (10 mg/kg). MMTY-PyMT mice were IV dosed with same dose of different nanoformulations of paclitaxel (10 mg/kg). At different time points, three mice were sacrificed to collect blood, tumor, and other normal organs. The drug concentrations in each tissues were measured using LC-MS/MS.

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Author Contributions
XL performed drug distribution in three cancer models and imaging analysis. HY performed imaging. BW and PS performed MS imaging analysis. MH, HZ, and FL performed animal experiments and LC-MS analysis. JP and NT helped to evaluate cancer cell efficacy in vitro. HH and WG helped to modify the nanoparticle formulations. SZ, MP, and MPP design the experiment and help to write the manuscript. DS design the experiments, analyze data and write the manuscript.

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REFERENCES


50. Socinski, M. A.; Bondarenko, I. N.; Karaseva, N. A.; Makhson, A.; Vynnichenko, I.; Okamoto, I.; Hon, J. K.; Hirsh, V.; Bhar, P.; Iglesias, J., Results of a randomized, phase III trial of nab-paclitaxel (nab-P) and carboplatin (C) compared with cremophor-based paclitaxel (P) and carboplatin as first-line therapy in advanced non-small cell lung cancer (NSCLC). *Journal of Clinical Oncology* 2010, 28 (18).


76. Maeda, H., Toward a full understanding of the EPR effect in primary and metastatic tumors as well as issues related to its heterogeneity. *Adv Drug Deliver Rev* 2015, 91, 3-6.
