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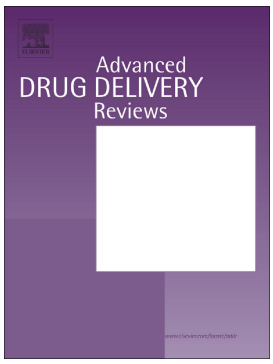


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# Translational Considerations in Nanomedicine: The Oncology Perspective

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**Running title:** Nanomedicine and oncology

## Abstract:

Nanoparticles can provide effective control of the release rate and tissue distribution of their drug payload, leading to major pharmacokinetic and pharmacodynamic changes vis-à-vis the conventional administration of free drugs. In the last two decades, we have witnessed major progress in the synthesis and characterization of engineered nanoparticles for imaging and treatment of cancers resulting in the approval for clinical use of several products and in new and promising approaches. Despite these advances, clinical applications of nanoparticle-based therapeutic and imaging agents remain limited due to biological, immunological, and translational barriers. There is a need to make high impact advances toward translation. In this review, we address biological, toxicological, immunological, and translational aspects of nanomedicine and discuss approaches to move the field forward productively. Overcoming these barriers may dramatically improve the development potential and role of nanomedicines in the oncology field and help meet the high expectations.

GRAPHICAL ABSTRACT[1]

## Sections:



[1] Adapted from Petersen, G. H. et al..*J. Controlled Release* 2016, 232, 255–264.

-4 Liposomes, the leading nanoparticle in clinical applications

-5 Targeted Nanomedicines

-6 Exploiting the interactions between the immune system and nanomedicines

-7 Imaging and Theranostics

-8 Clinical translation and optimization of the use of nanomedicines

-9 Future clinical landscape of nanomedicines in cancer

## 1. The challenge of cancer therapy:

Our understanding of the molecular processes underlying the pathophysiology of cancer cells has progressed enormously since the new century began. The extraordinary technological advances in genomics, the complete sequence of the human atlas genome and the characterization of gene mutations in various cancer types have led to the current view of a disease process defined by several key hallmarks driven by an underlying genetic instability of cancer cells [1]. Cancer is caused by somatic gene mutations followed by tumor progression along three major steps:

1. Increased proliferation and/or decreased apoptosis of tumor cells, causing an increase of tumor cell mass.

2. Invasion of adjacent tissues and switch on of angiogenesis

3. Metastatic spread from the primary tumor via blood vessels or lymphatics to distant organs, with formation of metastases. This is most frequently the process that causes death of the host.

In parallel to cancer growth and expansion, tumor cells undergo further genetic and phenotypic changes becoming resistant to many of the common cytotoxic drugs and developing mechanisms of escape from immune recognition. The extent and kinetics of these changes depends on the intrinsic characteristics of each tumor, interactions with the surrounding stroma and the selective pressure of anticancer therapies.

The clinical diagnosis of a tumor mass[2] requires usually a cluster of $10^9$ cells[3] (~10mm diameter). Most of these tumors are asymptomatic and identifying these small tumors would require screening with whole body imaging techniques, something that is not practical and not feasible for economic reasons. At the time of clinical diagnosis, most tumors have already covered 75% of their doubling cell expansion process. As a result, significant heterogeneity and phenotypic diversity are already present in most diagnosed cancers, posing a major therapeutic challenge due to the presence of cells with metastatic potential and multidrug resistance properties.

While surgery and radiotherapy are the main tools for treatment of localized disease, medical (drug-based) therapy is the established modality for treatment of disseminated cancer. Today medical therapy encompasses a broad array of agents with hugely different mechanisms of action, and includes chemotherapy (cytotoxic agents), hormonal therapy,

[2] Superficial skin tumors can be recognized sometimes when tumors contain smaller clusters of $10^7$ cells (~2-3 mm diameter).

[3] It is reasonable to assume that new techniques, based on proteomics or circulating tumor DNA, will be needed to safely break under the $10^9$ cancer cell mass diagnostic threshold.

biological therapy and immunotherapy. The latest addition is adoptive cell therapy exemplified by chimeric antigen receptor (CAR)-T cell therapy. Cell therapy is already at the edge of transplantation medicine although efforts are being made by pharmaceutical companies to impart to these advanced therapies with release specifications in line with pharmaceutical products. Medical therapy is applied in various settings to treat cancer:

- Primary (neo-adjuvant) treatment: Here, anti-tumor drugs are given prior to or concomitantly with potentially curative local therapeutic modalities (surgery or radiotherapy). In this setting, the primary tumor is present but there is no clinical evidence of distant metastases. This approach includes chemo-radiotherapy which is increasingly used to sensitize tumors to radiotherapy and enhance the anti-tumor effect.

- Adjuvant treatment: Adjuvant treatment is applied in patients with a high risk of micrometastases after surgical removal of the primary tumor. The adjuvant approach is a black box because all patients at high risk are treated without knowing which patients harbor viable metastases and which do not. Despite these shortcomings, it has been demonstrated statistically that adjuvant treatment can cure micrometastatic or subclinical, disease in a fraction of patients with some cancer types (breast cancer, colon cancer, and other tumors), who would not be amenable to cure if we wait for the disease to become macroscopic and clinically detectable before starting treatment.

- Treatment of metastatic disease: In most instances, including the most common types of cancer (breast, prostate, lung, and colon), medical drug therapy of cancer metastases is palliative, i.e. tumor regression and prolongation of survival can be achieved but cure is exceptional and most tumors ultimately recur and are lethal. Recent advances in immunotherapy using monoclonal antibodies that inhibit immune checkpoints and enable the switching on of the host anti-tumor immune response have opened a new era in cancer therapeutics. Complete and durable responses have been observed in a subpopulation of patients with some forms of advanced cancer, particularly melanoma and non-small cell lung cancer, treated with immune checkpoint inhibitors alone or in combination with cytotoxic chemotherapy [2, 3]. However, the role of the immune system in cancer progression and regression has not been fully elucidated, and the full clinical potential of the immunotherapy approach probably remains unexploited.

## 2. Cancer nanomedicine in 2020:

The field of nanomedicine encompasses the use of nanoparticles and macromolecules in the nanometric size range, mostly between 10 and 200 nm, that enable unique and complex interactions with the biological milieu. In most instances, nanomedicines are drug delivery

systems consisting of a carrier and an associated drug, but in some cases the nanoparticle is the active agent itself as is the case with gold nanoparticles that can destroy tumors by a photothermal effect [4]. Formulating a single molecule drug, several angstroms across, into a nanoparticle packed with thousands of drug molecules and with ~1 million-fold greater volume is a tremendous pharmaceutical challenge with major pharmacological implications. While nanomedicine is basically a technology, there is also an important and specific science side of nanomedicine due to special and complex interactions of nano-size drug delivery systems with the biological milieu that result in unique pharmacodynamic effects.

The last couple of decades have witnessed signficant progress in the synthesis, engineering and characterization of nanoparticles for therapy mainly for cancer therapy and diagnosis. The FDA has approved several nano-drug products, mostly liposomes for intravenous administration. New promising candidates are in different stages of clinical trials. Nanotechnology research has involved different types of nanomaterials [5], based on organic components (lipids, polymers, cell-derived vesicles), inorganic components (metals, carbon-based, mesoporous silica) and even gas-filled vesicles (microbubbles), with the common goal of improving drug delivery and cancer treatment [6]. The success of these nano-drugs stems mainly from a reduction of the life-threatening toxicities associated with some of the anti-cancer agents delivered by nanomedicines. Nevertheless, the clinical use of nano-drugs has resulted so far in a limited improvement in the overall survival of patients [7]. The impact of nanoparticle-based drugs remains under-exploited with a modest presence in the field of cancer drug development. Nanomedicine is an attractive tool for reformulating some old drugs or for delivering undruggable molecules in a convenient form of administration, but a substantial impact of nanomedicine in cancer therapy requires developing products with significant added value, either greater safety or greater efficacy, over the established technologies.

Nanoparticles and polymeric macromolecules are the most important tools of nanomedicine [8]. Doxil, also known as pegylated liposomal doxorubicin (PLD)[4], was the first nanoparticle-based cancer chemotherapeutic approved by the FDA [9, 10]. Thus far, PLD together with nanoparticle albumin-bound paclitaxel (NAB-paclitaxel)[5] [11] are considered the cancer nanomedicines that have made the main clinical contribution. Both PLD and NAB-paclitaxel have been approved as single agent or in combination therapy for a number of important indications including breast and ovarian cancers, multiple myeloma, and Kaposi sarcoma for PLD; and breast, pancreatic, and non-small cell lung cancers for NAB-paclitaxel.

[4] Marketed under the trade names of Doxil and Caelyx by Janssen Pharmaceuticals and as Lipodox by Sun Pharmaceuticals.
[5] Marketed under the trade name of Abraxane by Celgene.

Nanoparticles can improve the delivery of chemotherapeutics by controlling release rate of the active agent and by changes in drug biodistribution that will relatively spare sensitive tissues while enhancing drug deposition in tumors by the Enhanced Permeability and Retention (EPR) effect [12, 13], a phenomenon referred as passive targeting (Fig. 1). Most of the nanopharmaceuticals approved for clinical use in cancer treatment are liposome-based and belong to the non-targeted or passive targeted category (Table 1). Active targeting implies a targeting component acting as specific ligand for a receptor expressed in cancer cells and is discussed below in section 6 (**Targeted nanomedicines**).

EPR is a critical requirement for nanoparticle transport from the blood stream into tumors [12, 13]. Abnormal blood vessels, large fenestrations, discontinuous basement membrane, high microvascular permeability and defective lymphatic drainage are frequent features of tumor-associated neoangiogenesis, in contrast to the normal blood vessels of non-malignant tissues [14] (Fig. 2). This cancer hallmark is the pathophysiologic basis for EPR.

While EPR is observed consistently in many experimental tumor models, large variations have been observed in human cancer as reviewed by Man et al. [15]. Back in 2001, Harrington et al. observed a large inter-patient variation between 2.7 and 53.0% ID/kg, based on scintigraphic studies with $^{111}$In-labeled stealth (long-circulating) liposomes and volumetric estimates of tumors [16]. Direct contributing factors to EPR variability include tumor type, tumor size, and tumor site (primary versus metastatic tumors). Mechanistically, the underlying factors of EPR variability are related to the microanatomy of tumor blood vessels, the presence and number of tumor-associated macrophages (TAM), and the tumor interstitial fluid pressure (IFP).

There are instances of tumors or metastases displaying weak or ineffective EPR effect, such as when the tumor blood supply is derived by a process known as co-option of normal blood vessels which results in blood vessels with reduced permeability and responsiveness to anti-angiogenic treatments [17].

The prevalence of TAM is high in many tumors and may entail a poor prognosis [18]. TAM have been shown in several studies to be the main cellular reservoir of nanoparticles that reach the tumor microenvironment [19, 20]. Indeed, it has been shown, using a PET-imaging tool of TAM, that TAM-rich tumors accumulate several hundred-fold more polymeric nanoparticles than TAM-depleted tumors [21].

High IFP develops in most large tumors, above a threshold size, as a result of defective lymphatic drainage and is a major obstacle to drug delivery in general and to nanodrug delivery in particular [22]. High IFP may shut off the convective transport of fluid from the intravascular to the extravascular compartment which is greatly important for the extravasation and penetration of nanoparticles into the tumor tissue. Adding this to the solid stress caused by tumor growth and desmoplastic reaction will lead to collapse of tumor blood vessels, decreased perfusion, hypoxia and necrosis [23]. Experimental attempts

at reducing IFP in tumors with anti-angiogenic therapy suggest that nanomedicine delivery can be improved but only for particles smaller than 100 nm [24].

In Kaposi sarcoma skin lesions, a tumor with high vascular permeability and high EPR, radiolabeled Stealth liposomes deposit in large amounts (Fig. 3A). In good correlation with the imaging findings, biopsies in these patients show high concentrations of PLD, 16-fold greater than in normal skin in average (Fig. 3B-C) [16, 25]. Moreover, the high response rate of Kaposi sarcoma to relatively low doses of PLD [26] suggests that EPR makes an important contribution to the antitumor activity of nanodrugs by increasing their tumor drug levels. Yet, while selecting tumors with high EPR for treatment with nanodrugs is pharmacologically sound, we cannot discard that low-EPR tumor will still respond to nanodrugs better than to free drugs.

Th mechanism of EPR is still debated and is probably complex and multifactorial. Extravasation of nanoparticles through gaps between endothelial cells [27] driven by convective flow and diffusion has been the central paradigm, but a recent paper has provided evidence for a major contribution of transcytosis with pegylated gold nanoparticles in 4 different mouse tumor models [28]. Back in the 1990's, a combination of extravasation and transcytosis was reported to mediate the EPR effect in a model of Kaposi sarcoma using pegylated colloidal gold liposomes [29]. Importantly, extravasation may not be a steady process but rather a dynamic one with vascular burts of convective transport through vents or transient gaps [30]. It has been further shown that EPR can be enhaced by bursts of macrophage activity afte radiation [31]. EPR variability is discussed further in section 7 (Imaging and theranostics).

## 3. Translational challenges

In a thorough review on strategies to improve the translation success of cancer nanomedicines, Van der Meel et al. [32] proposed 4 directions: patient stratification and selection, rational drug selection rather than opportunistic choices, combination and multimodal therapies for synergistic effects, and empowering immunotherapy. While some of these points will be addressed in later sections, we will focus first on the basic clinical regulatory approach in which the two issues that most matter for the acceptance of a nano-pharmaceutical product are: Is the safety profile of the nanomedicine better than the standard treatment? Is the efficacy of the nanomedicine superior to the standard treatment? To achieve these objectives, the nanoparticle engineering strategy has to meet several translational goals:

a. Stable association of drug and carrier in circulation: Determining stability of the carrier in circulation is relatively easy to check by looking for free drug in *in vitro* plasma stability assays or *in vivo* during pharmacokinetic testing. High stability is critical to keep the drug payload in association with the carrier when a change in

tissue biodistribution is sought. When the main purpose is to achieve slow drug release from the central compartment, what is needed is a controlled rate of release.

b. Enhanced drug delivery to tumors: For this to occur, first, the nanodrug has to remain long in circulation and maintain high plasma levels, thereby increasing the number of passages through the tumor microvasculature and the efficiency of extravasation (i.e., number of particles moving into the tumor compartment per unit of time by diffusion and convection). Second, the nanoparticle size has to allow extravasation across tumor blood vessels, but to prevent extravasation across normal blood vessels, to spare sensitive tissues (e.g., heart muscle, nervous system, gastro-intestinal mucosa), and to avoid loss through renal glomerular filtration. Up to 400 nm-diameter liposomes have been shown to extravasate and concentrate in tumors by exploiting the EPR effect [33]. However, given the pharmaceutical necessity for sterile filtration of all systemically administered nanoparticle products, the optimal size window that will take advantage of the permeability range in normal and tumor blood vessels appears to be between 20 and 200 nm. It should be noted that sharing similar systemic PK of a nanodrug does not necessarily lead to the same extravasation efficiency, since the latter is substantially affected by the phenotypic characteristics of the tumor microenvironment [34].
c. Release of active drug in tissues at a suitable rate for anti-tumor activity. Examining the release rate of drug from carrier in tissue is very challenging and sometimes can only be inferred from pharmacodynamic observations indicating drug bioavailability. Even then, the pharmacodynamic read-outs do not always reflect the kinetics of the process of drug release in tissues. Animal models are helpful to screen nanomedicines, but the kinetics of nanomedicines are species dependent as indicated by the large inter-species variations in circulation half-life and, therefore, animal results are not always generalizable to humans. While there is clear evidence of bioavailability for PLD, this is not the case for SPI-077, a cisplatin-containing liposomal nanodrug, whose clinical development failed due to poor bioavailability in the tumor site and lack of anti-tumor activity [35, 36]. Release rate of drugs from nanocarriers vary among tissues and is affected by the presence of tissue-resident macrophages. As mentioned before, a significant fraction of tumor-homing nanoparticles is taken up by TAM, and it is well accepted that endocytosis results in faster breakdown of nanoparticles than degradation taking place in extracellular fluids. Uptake of nanodrugs will inhibit Kupffer cell and other macrophage activity [37], but also creates a reservoir of drug in the TAM compartment with gradual release of free drug that can diffuse and inhibit neighboring tumor cells [38, 39]. Therefore, targeting the TAM compartment, while minimizing systemic RES damage, will help potentiate the antitumor effect of some nanodrugs.

Successful control of these parameters in the nano-formulation will spare toxicity to normal tissues and boost the antitumor effect, thereby enabling an overall increase of the

therapeutic index. Many nanomedicines have failed to meet these requirements because of short circulation time, poor drug retention, or insufficient drug release. Yet, other nanomedicines have been able to make a positive clinical contribution despite only minor changes in drug pharmacokinetics (PK). This is the case of NAB-paclitaxel which avoids the acute toxicities associated with Cremophor EL® vehicle used in solvent-based paclitaxel and has been found useful in various indications [40].

A number of technology issues have to be addressed early in product development for successful translation. During the basic research phase, each nanomedicine must be optimized with regard to its proposed clinical use, route of administration, projected dose, and frequency of dosing. There are several empirical check lists and stop-go checkpoints common to all nanomedicines. For nanomaterials, they include physico-chemical characterization, biocompatibility, biodegradability, and availability of GMP sources. The toxicity risk imposed by some nanomaterials has to be weighed against the potential drug delivery advantage it conveys when formulated in a specific nanoparticle. For instance, while mesoporous silica nanoparticles are very attractive and robust systems for controlled drug delivery [41], silica and mesoporous silica nanoparticles display problematic toxicity when injected intravenously [42]. Yet, reducing the size of functionalized silica particles to <10 nm to allow glomerular filtration results in an apparently safe and effective approach to cancer targeting [43, 44].

Since most of the nanomedicines are used by the parenteral route, sterile filtration will be required in the manufacturing process, thus imposing a particle size limit of 200 nm. In addition, if the nanomedicine is to be configured with a targeted ligand and/or with a chelating agent for imaging theranostic applications, it is important to do it from the beginning since even small modifications may change the *in vivo* stability and PK [45].

Regarding the active pharmaceutical ingredient, it is important to consider upfront its potency in relation to the maximal payload achievable. Low potency drugs even at optimal payload may require the infusion of a prohibitive amount of nanoparticle mass to deliver a pharmacological dose. Designing a robust manufacturing process that can ensure a stable product with reproducible drug-to-nanoparticle ratio and minimal contamination with free drug is critical.

A simplified flow chart for development of a nanomedicine is presented in Fig. 4.

## 4. Liposomes, the leading nanoparticle in clinical applications:

Liposomes are among the most frequently used nanoparticle systems for parenteral delivery of drugs, particularly for cancer chemotherapeutic agents. Polyethyleneglycol-coated (pegylated) liposomes have a prolonged circulation time in the blood stream, which results in enhanced accumulation in tumors by the EPR effect.

Liposomes and other nanocarriers can be classified into 4 categories as proposed by Hsu and Huang [46] based on the drug release rate in circulation and the clearance by the reticulo-endothelial system (RES), also referred to as mononuclear-phagocyte system (MPS). As shown in Fig. 5, class 2 are the most attractive nanocarriers since they are highly stable and have low affinity for the RES. This results in a long-circulating nanoparticle with an intact drug payload, and effective control of drug delivery and tissue distribution by the nanocarrier. Among the clinically approved nanomedicines, PLD is a good example of this class of formulations. The circulation half-life is long, in the range of 2 to 3 days and the leakage of doxorubicin is insignificant with undetectable or very low concentrations of free drug (~1,000-fold lower than liposomal doxorubicin) [47].

There are several reasons for liposomes and lipid nanoparticles to be the most well-accepted nanotechnology for clinical applications. Liposome building materials are biocompatible and biodegradable and GMP sources are available and well characterized. Manufacturing at upscalable commercial levels is feasible and with well-developed analytical protocols. There is large amount of long-term toxicology data on liposomes and a good understanding of their PK and to some extent biodistribution in humans. Thus, regulatory-wise, liposomes are well ahead of other nanoparticles. This is in large measure due to the fact that liposome-based doxorubicin and amphotericin B were the first nanodrugs to be approved by regulatory bodies.

The attempts to formulate anthracyclines in liposomes began nearly 4 decades ago (reviewed in [10, 48]) with a simple strategy: shifting drug biodistribution to spare the heart muscle from anthracycline-induced cardiac toxicity and maintaining the antitumor effect. Later, it was recognized that long-circulating, small unilamellar liposomes accumulate in tumors in high amounts [49]. Subsequently, two important technological developments, pegylation of liposomes [50, 51] and remote loading of cationic amphiphiles [52] led to the PLD-Doxil formulation [9, 53]. The reduction of cardiotoxicity when doxorubicin is compared to PLD is huge with more than a 3-fold increase in the maximal cumulative dose [48, 54] and is likely to result from decreased drug exposure of the heart muscle (Fig. 6A). In addition, the passive tumor targeting effect of these long-circulating liposomes conferred by the EPR effect is highly significant and reaches values ≥ 10% of the injected dose when normalized per unit tumor weight (grams for mice, kg for humans)[16, 55, 56]. These values are several fold greater than the drug levels obtained with liposomal drugs in extra-RES normal tissues and with free drugs in tumors based on animal and human data [36, 47, 57-60]. Yet, it is important to take into account that particle size, circulation half-life, dose (Fig. 6B), and tumor size (Fig. 6C-D) significantly affect tumor uptake [19, 61, 62]. This highlights the risks of drawing conclusions from inter-study comparisons in different tumor models and with nanoparticles of different and, often, suboptimal characteristics [63] that may result in an underestimation of the potential of nanomedicine in cancer drug delivery.

Besides Stealth liposomes, other nanoparticles have achieved long half-lives and high tumor uptake. Core-crosslinked polymeric micelles and other polymeric micelles are attractive pharmaceutical products with solid preclinical data and are currently in early clinical testing for the delivery of docetaxel and platinum-based drugs [64-66].

Triggered release of liposomal drugs is another area with extensive research aiming at clinical translation. Thermosensitive liposomes that respond to hyperthermia with bursts of drug release have been developed into a clinical formulation containing doxorubicin known as Thermodox®. A phase 1 clinical study showed a significant increase in tumor drug levels when focused ultrasound was applied to heat the tumor [67]. Experimentally, Thermodox delivers more drug to tumors than PLD upon hyperthermia but, on the down side, it has a faster clearance and a narrow time window for exploiting its efficacy [68]. While the initial pivotal study with Thermodox failed to achieve its endpoints, another phase 3 study combining Thermodox with radiofrequency ablation (RFA) for treatment of Hepatoma is still ongoing and final efficacy results are awaited[6].

## 5. Targeted nanomedicines

Tumor cells overexpress a variety of surface receptors that play an important role as catalysts of tumor growth. This area is of particular relevance to cancer targeting. Receptor profiling of tumors [6] with overexpressed receptors on the tumor cell membrane, may offer a potential Trojan horse for targeting specific ligands or antibodies and delivering a cytotoxic drug cargo. One of the best examples of a successful clinical translation of this approach is the antibody-drug conjugate known as T-DM1 which combines Trastuzumab, an anti-Her2 antibody, with emtansine, a potent and highly toxic chemotherapeutic, and has conferred a significant disease-free survival advantage to patients with Her2-positive breast cancer .

Targeted delivery of a large drug payload to cancer cells via ligand-receptor specific interaction is probably the most cherished goal of nanomedicine. A comprehensive and in-depth review of this subject has been recently published [69]. Active targeting of nano-based drugs requires the coupling of ligands to the surface of the nanoparticle directed to a cancer-specific cell surface receptor, a process that should be differentiated from passive targeting achieved solely by the EPR effect. Active targeting would allow internalization and intracellular delivery of the drug cargo into target cells, which is the holy grail of nanomedicine. The active targeting approach requires a more complex formulation design: the ligand has to be anchored on the external surface of the nanoparticle for which it may need to be chemically modified, the number of ligands per particle has to be carefully controlled, and the ligand may modify the PK of the nanoparticle reducing circulation half-

[6] Celsion Reports Unanimous Independent Data Monitoring Committee Recommendation to Continue the Phase III OPTIMA Study of ThermoDox® in Primary Liver Cancer. November 4, 2019 8:00 AM |GlobeNewswire| CLSN.

life, possibly affecting EPR-based tumor uptake [45], and increase the risk of immune reactions, particularly if it is a protein or a peptide.

An important advantage of targeted nanocarriers over ligand-drug bioconjugates is the delivery-amplifying effect which can convey to a tumor cell the full drug payload of a liposome to the target cell per single ligand-receptor interaction. In addition, the target-specific avidity of nanoparticles can be significantly enhanced by orders of magnitude through multivalent interactions, particularly with small molecule ligands [69].

Examples of actively targeted nanomedicines clinically tested include MM-302, a Her2-targeted Doxil liposome [70], and BIND-014, a PSMA-targeted docetaxel polymeric nanoparticle [71]. Unfortunately, the clinical development of both products has been discontinued: MM-302 did not provide any clinical advantage over the chemotherapy control arm given to breast cancer patients (Hermione study) [72], and BIND-014 circulation time in humans is relatively short ($t_{½}$ = ~ 6 hours) and probably does not enable significant EPR-mediated accumulation in tumors. For comparison, CPC634, another polymeric (non-targeted) docetaxel nanoparticle currently in clinical studies, has a half-life of 33 hours [73] at the same docetaxel dose level of 60 mg/m$^2$ as BIND-014 indicating greater stability and drug retention in circulation.

Targeting to the folate receptor (FR), which has been found to be upregulated in multiple cancer types and in inflammatory macrophages [74, 75], has been extensively investigated with small drug conjugates, polymers and nanoparticles with promising results. However, the rate limiting step for tumor localization of targeted nanomedicines as well as non-targeted ones, when administered by the systemic route, remains EPR-dependent extravasation [76]. Only thereafter the ligand can interact with the tumor cell receptor and confer a pharmacological advantage. Unfortunately, folate ligands shorten circulation time in experimental models reducing the efficiency of the EPR effect and tumor targeting [45, 77]. Intra-cavitary therapy could be an alternative approach for exploiting the translatability of targeted nanomedicines with higher probability of success, since this route (intra-vesical, intra-peritoneal, other) exposes directly nanomedicines to tumor cells [78-81]. An example of the pharmacological impact of a targeted nanomedicine in an intracavitary tumor model is presented in Fig. 7 [82, 83].

## 6. Exploiting the interactions between the immune system and nanomedicines

Nanoparticles interact with the immune system although the extent of interactions may vary depending on the physical and chemical characteristics of the nanoparticle such as composition, size, and shape [84]. The primary mechanism of clearance for the majority of intravenously administered nanoparticles is through internalization by splenic macrophages and hepatic Kupffer cells. Clinically approved nanoparticle-based therapies have interactions with the host immune system that could potentially affect drug pharmacokinetics and result in significant consequences for nanoparticle drug tolerability and efficacy. Notably, the rate

of PLD clearance in cancer patients was observed to have a strong correlation with markers of the RES functionality such as the number of circulating monocytes and their phagocytic capacity, supporting the role of the RES in the pharmacology of nanoparticle drugs. Similar correlations between monocyte number or function have been reported in rodent and canine models treated with other pegylated liposomal formulations such as liposomal belotecan (S-CKD-602) and liposomal cisplatin (SPI-077) [85]. Thus, uptake and sequestration of lipid nanoparticles by the RES is viewed as a major obstacle limiting the circulation half-life of the drug which consequently also diminishes tumor accumulation of nanoparticle-mediated drugs that act directly on tumor cells, such as cytotoxic chemotherapy. Formulation strategies aimed at avoiding RES uptake of nanoparticles have been successful at delaying particle clearance from circulation and extending circulation time, but ultimately the majority of the systemically administered nanoparticles will end up in the RES.

Rather than trying to avoid the RES, it may be advantageous to leverage this propensity for nanoparticles to be internalized by phagocytes to target the RES. For instance, uptake of iron-based nanoparticles (e.g., Ferumoxytol) by monocytes and macrophages has been successfully exploited for lymph node imaging and diagnosis of micrometastases in the sentinel lymph nodes of prostate cancer patients [86]. Other successful examples of RES targeting include highly negatively-charged liposomes for delivery of doxorubicin in the treatment of diffuse hepato-splenic metastatic spread in a lymphoma model [87], and liposomal delivery of clodronate for depletion of blood monocytes and macrophages, and tentatively of TAMs [88]. This latter strategy was used to target clodronate to macrophages for the ablation of TAMs that promote cancer progression [18]. However, liposomal clodronate actually diminished the anticancer efficacy of concomitantly administered anti-cancer nanomedicines [39], a result that can be explained by the use of a liposome formulation with short half-life and poor tumor accumulation coupled with the low potency of clodronate, and the fact that TAMs often contribute to the anti-tumor effect acting as tumor drug reservoirs after uptake of nanomedicines [39]. Recently, long-circulating liposomes were exploited for the delivery of alendronate, a potent amino-bisphosphonate, to functionally polarize TAM toward an antitumor phenotype, rather than to deplete them, and significant inhibition of tumor growth was observed in an immunocompetent murine cancer model [89].

Immunotherapy may benefit from the use of nanoparticles to target antigen-presenting cells and T cells in the spleen and lymph nodes, the major sites of naïve T cell priming and activation against antigens [90]. The tumor-draining lymph nodes, also known as the sentinel lymph nodes, are also the first site of tumor metastases making them an attractive target for treatment or diagnostic imaging of occult metastases [91].

In addition to interactions with the RES, it is well established that nano-carriers interact with serum proteins such as immunoglobulins (including IgG and IgM) and circulating complement proteins, which form a protein corona on the nanoparticle. The nature of the

protein corona impacts opsonization of the carrier and clearance by the RES. It may also lead to formation of immune complexes, generation of an immunogenic epitope, and modulation of immune responses [92-94]. Moreover, the protein corona can interfere with targeting functions of active targeting molecules such as antibody fragments conjugated to the surface of the nanoparticles [95]. The composition of the protein corona is dynamic and highly variable, depending on both the fluctuations in host circulating proteins and the physical and chemical characteristics of the nanoparticle. Hence, *in vitro* studies and studies in non-diseased animals may not fully characterize the protein corona nor the biological impact of liposomal drugs that are intended for use in cancer patients. Another important consequence of liposome interaction with serum proteins is activation of the complement cascade, generating complement cleavage products C3a, C4a, and C5a, that are anaphylatoxins which can stimulate immune cells to release inflammatory mediators such as histamine. This can result in complement activation-related pseudoallergic reactions (CARPA) in canine and swine models. Several formulations of nanoparticles in clinical use (e.g.: PLD, DaunoXome, AmBisome) are known to cause hypersensitivity reactions that are consistent with CARPA. Clinically, the development of acute infusion reactions in cancer patients receiving PLD have been reported to correlate the levels of complement cleavage products in the peripheral blood [96]. Therefore, interactions between circulating serum proteins and nanoparticles can adversely affect the PK and tolerability of nanoparticle-mediated drugs.

Various formulation strategies have been developed to reduce protein opsonization on nanoparticles. One of the most widely used approach is pegylation; this has been shown to improve stability of the nanoparticle in plasma and increase circulation time, requisites for effective tumor targeting via the EPR effect. However, these approaches do not abolish interactions between nanoparticles and the immune system. In addition, recent reports suggests that PEG itself may have immune modulatory effects. Several groups have demonstrated that the first dose of a systemically administered pegylated nanoparticle induced an adaptive immune response characterized by the production of IgM antibodies against PEG which enhanced immunogenicity and clearance of the second dose of nanoparticles in preclinical models. However, the clinical relevance of this “accelerated blood clearance” (ABC) phenomenon is unknown since this has not been observed in patients [97].

Interestingly, it was recently shown that nanoparticle-induced complement activation could promote tumor growth in murine tumor models, through C5a-receptor mediated recruitment and also activation of immunosuppressive myeloid cells. Nonetheless, these nanoparticles were designed to robustly activate the complement cascade [98] while the opposite stratey, to design nanoparticles with minimal complement activation, is the goal of most nanoparticle drug formulations. Moreover, it is unclear whether clinically approved nanoparticle carriers that induce complement activation in the peripheral blood also induce complement activation within the tumor tissue, and how this impacts cancer progression or

regression. New data with a nanoparticle similar to the PLD carrier, showed that pegylated liposomes have the potential to enhance tumor growth in immune competent mice bearing implanted syngeneic tumors [99]. This was associated with diminished cytokine production in TAMs and tumor infiltrating cytotoxic T cells, and decreased tumor antigen specific immune responses, suggesting inhibition of antitumor immunity. Furthermore, vasculature density in tumor tissue was significantly increased, suggesting enhanced angiogenesis. Based on these data, it seems possible that nanoparticle-induced immune modulation could lead to suboptimal efficacy of the nanoparticle-encapsulated drug. This could be one reason why many of the clinical studies with nanoparticle-delivered drugs have failed to show a significant improvement in efficacy (as measured by progression-free or overall survival) over the non-nanoparticle comparator treatment [100]. A possible contributor to the disparate efficacy findings between preclinical and clinical studies are the baseline differences in PEG exposure. While most cancer patients have pre-existing antibodies against PEG due to the use of PEG as a common ingredient in hygiene and cosmetic products [101], experimental animals are largely PEG-naïve at baseline.

Historically, preclinical cancer drug development has relied heavily on the use of rodent tumor models with major immune defects and rarely were systematic and extensive *in vivo* immunological studies part of the drug development paradigm. The interactions between nanoparticle drug carriers and the immune system have generally been viewed as secondary to the antiproliferative effects of the drug cargo. The immune system is a key player in cancer pathophysiology and also a key player in nanoparticle drug pharmacokinetics. One major implication of the interactions between nanoparticles and the immune system is that preclinical studies should incorporate immune competent models of cancer along with *in vivo* studies assessing innate and adaptive immune responses in order to gain accurate insight and tools to fully assess the clinical potential of nanoparticle-based therapies (Fig. 8).

## 7. Imaging and Theranostics

The early integration of imaging methods with nanomedicine is likely to lead to more efficient preclinical development and clinical translation, as well as improved therapeutic outcomes. Firstly, being able to visualize and quantify the biodistribution and PKs of nanomedicines - at the whole-body level - provides invaluable information at the early and late stages of their preclinical development. Furthermore, taking into account that patient and disease heterogeneity is prevalent in cancer (*e.g.* EPR heterogeneity discussed above), imaging can play a powerful theranostic role in clinics, allowing the identification and selection of the patients that are most likely to respond to the treatment with nanomedicines, facilitating what has been termed as “personalized nanomedicine” [102-104].

Several imaging techniques are available that can provide such information in the clinical setting. However, only nuclear imaging techniques such as positron emission

tomography (PET), single-photon emission computed tomography (SPECT), and to a lesser extent magnetic resonance imaging (MRI) and X-ray computed tomography (CT) have the key properties required. These properties are:

i. **Tissue penetration of the imaging signal**: The imaging signal should not be affected by its location within the body. In this aspect, nuclear, MR and CT imaging have excellent tissue-penetration. This is in contrast to other techniques such as optical imaging, where the signal is not capable of penetrating deep into tissues.
ii. **Whole-body imaging capabilities**: The technique should allow imaging the whole body. Here PET, SPECT, and CT have a clear advantage over MRI, as performing whole body MRI is more challenging than with PET, SPECT or CT. PET/CT hybrid scanners providing fusion images with functional and anatomical information have become a leading diagnostic tool in oncology. Recently, the advent of the total-body PET scanner for clinical use is likely to make a tremendous impact, allowing high spatio-temporal resolution imaging of the whole body using very low doses of radiation (up to 40 times lower compared to current clinical PET) [105].
iii. **Quantification**: The imaging signal must be quantifiable to provide accurate information of the concentration of nanomedines at a given time. Quantification of imaging signals with PET is significantly more accurate than with of MRI or CT. This is mostly due to MRI and CT having an endogenous signal from tissue and hence, low signal-to-background ratios. In addition, accurate quantification of MR and CT imaging signals often requires a preliminary, contrast agent-free image to be acquired prior to the actual contrast-enhanced scan.
iv. **Sensitivity**: Sensitivity in imaging terms is the amount of imaging/contrast agent required to obtain a detectable and quantifiable imaging signal. Nuclear imaging has a clear advantage over MRI and CT as the amount of imaging/contrast agent required for nuclear imaging techniques is *ca.* $10^6$ times lower than for MRI and CT (*e.g.* micrograms in PET/SPECT *vs*. grams in MRI and CT). This allows the administration of subtherapeutic microdoses of the nanomedicine ($1/100^{th}$ of the therapeutic dose) when using PET/SPECT, which is a clear advantage for the theranostic application of these imaging techniques. Furthermore, the new total-body PET technology mentioned above, with its increased sensitivity over current PET, will facilitate this aspect even further.

Taking all these properties into account, it is not surprising that most clinical studies where imaging was included in the evaluation of nanomedicines in patients have chosen nuclear imaging techniques (*vide infra*), followed by MRI; as we recently reviewed [15].

Labelling nanomedicines to be tracked using nuclear imaging techniques requires the incorporation of a radionuclide into its structure, and this has implications in terms of clinical translation. In the case of liposomes, we have recently performed a comprehensive review of the different methods available for radiolabeling, their consequences for clinical translation, as well as applications in nanomedicine [106]. If the goal is to image an already clinically approved product such as the PLD stealth liposome, care must be taken to not modify the physicochemical and surface properties of the original product. In this respect, radiolabeling the intraliposomal core of the liposome is preferred, as chemical modification of the surface (*i.e.* phospholipid bilayer in the case of liposomes) can have significant

consequences on the *in vivo* behavior. For example, a direct comparison between different radiolabeling methods for stealth PEGylated liposomes (membrane *vs.* core radiolabeling) in an *in vivo* animal model of inflammation found significant differences in the levels of liver uptake, which was higher for the surface-modified method [107]. It is possible that this is a consequence of the presence of a chelator (DTPA) on the surface of the liposome, required for membrane radiolabeling, leading to increased interaction with the RES.

Intraliposomal labelling can be achieved by several methods [106], and unlike surface-based methods, it is less prone to result in changes in the physicochemical and/or surface properties of the original product. In this approach, the imaging label is highly protected from external factors that can contribute to low *in vivo* stability. In order to achieve intraliposomal radiolabeling, the imaging label has to be able to cross the lipid bilayer. This is frequently achieved using ionophores, which are trans-lipid membrane transporting molecules widely used for cell labelling in the field of nuclear medicine. The most widely used ionophore is 8-hydroxyquinoline, a clinically approved molecule commonly known as oxine. Using 8-hydroxyquinoline or isomers thereof, several radiometals such as $^{111}$In [108-110], $^{67}$Ga [111], $^{64}$Cu [112, 113], $^{89}$Zr [113], and $^{52}$Mn [113-115] have been incorporated into liposomes. All these reports highlight the versatility and efficiency of the hydroxyquinoline platform for this purpose. Given the metastability of the radiometal-ionophore complexes formed, the intraliposomal space must contain a radiometal chelator for achieving effective retention inside the liposome and *in vivo* stability. This has commonly achieved by encapsulating well-established chelators such as DTPA (for $^{111}$In), DFO (for $^{67}$Ga or $^{89}$Zr), or DOTA (for $^{64}$Cu or $^{52}$Mn). However, if the aim is to radiolabel a preformed liposome, encapsulation of a chelator represents a significant modification of the formulation and hence a preclinical clinical translation hurdle. An alternative to the encapsulation of exogenous metal chelators is the use of the already-loaded intraliposomal drugs, many of which have significant metal-binding chelating properties [113]. Using this approach, we have shown that preformulated liposomes encapsulating widely used and clinically-approved drugs such as aminobisphosphonates (*e.g.* alendronate) [113, 116], anthracyclines (*e.g.* doxorubicin) [113, 115], or glucocorticoids (*e.g.* methylprednisolone) [117], can be efficiently radiolabeled ($^{89}$Zr, $^{64}$Cu, $^{52}$Mn, $^{111}$In) using hydroxyquinoline ionophores and imaged *in vivo* with high stability. For example, using $^{89}$Zr-, $^{64}$Cu-, and $^{111}$In-labelled PEGylated liposomal alendronate (PLA) we have demonstrated the long-circulating properties of this formulation, as well as high EPR-mediated uptake (*ca.* 10-15% ID/g) in breast and ovarian tumors (in immunocompromised mice - MTLn3, MDA-MB-231 and SKOV3) (Fig. 9A). Interestingly, high levels of uptake have been observed not just in primary tumors, but also in metastases, namely in lymph nodes and lungs (Fig. 9B) [113].

Surface radiolabeling, despite the potential disadvantages discussed above, is a very valuable approach for the preclinical imaging of nanomedicines and for the development of companion diagnostics for clinical nanomedicinal products. In a notable example of the latter, Perez-Medina *et al.* developed a PEGylated liposomal formulation (with similar physicochemical properties to PLD) containing deferoxamine (DFO) as part of the phospholipid bilayer on the surface [118]. DFO is an excellent chelator for the PET radionuclide zirconium-89 ($^{89}$Zr) that has a decay half-life of *ca.* 78h, making it the ideal

radiometal-chelator couple to track long-circulating nanomedicines such as PLD that commonly have circulating half-lives of ~2-3 days in humans [47]. Using this $^{89}$Zr-liposome and PET imaging the authors carried out imaging-therapy studies using PLD in a mouse model of breast cancer. Several important findings were found (Fig. 10A-B): *(i)* PET signal in tumors correlated with doxorubicin concentration; *(ii)* a high variability of PET signal/doxorubicin concentration in this tumor model, in line with the high EPR heterogeneity often found in humans, and *(iii)* a possible correlation of high PET signal with tumor growth delay. A similar observation was made by Karathanasis et al. in a different mous model with PLD and a liposome containing X-ray contrast (Fig. 10C-D) [119]. This highlights the interesting possibility of using a single companion diagnostic agent when nanomedicines rely on a common tumor-uptake mechanism such as the EPR phenomenon.

Despite the many reports of integrating imaging methods into the preclinical development of nanomedicines, very few clinical studies have exploited this approach [15]. Most of these studies have used nuclear imaging techniques for the reasons discussed above, with a tendency towards using PET and liposomes as the main nanomedicine used for clinical drug delivery. Notably, despite reports claiming that the EPR effect does not lead to tumor targeting in patients [120], several independent clinical studies have provided substantial evidence that EPR occurs in humans, and leads to significant accumulation of nanomedicines in tumors by passive targeting, albeit with large variation depending on cancer type and high interpatient and intrapatient heterogeneity [15, 121].

A landmark study in this area was carried out by Harrington *et al.* [16]. Using PLD-like $^{111}$In-labelled PEGylated stealth liposomes (intraliposomal labelling using encapsulated DTPA), the authors confirmed the long circulation half-life of this system in patients ($t_{1/2}$ = 76h), matching that of PLD. Remarkably, out of the 17 patients imaged, 15 of them showed clear high accumulation of the $^{111}$In-liposome in different types of solid tumors in addition to a patient with Kaposi sarcoma (Fig. 3), with doses as high as *ca.* 3.6 % of the injected dose per tumor and 53.0% ID/kg tumor based on the imaging-estimated tumor volume. Heterogeneity of tumor uptake was also demonstrated by the high variability between different tumor types (lung cancer, high-grade glioma, advanced breast cancer, cervix cancer, or squamous cell HNC), and even within the same tumor type. The authors proposed the use of such imaging approach to predict liposomal drug levels in tumors of patients.

More recently, a notable study has shown the benefits of integrating PET imaging with the development and clinical evaluation of nanomedicines. In this case, the authors developed a intraliposomal radiolabelling technique for HER2-targeted liposomal doxorubicin (MM-302) with the PET radionuclide $^{64}$Cu ($t_{1/2}$ = 12.7h) [122]. This targeted nanomedicine had shown potential for increased doxorubicin delivery to HER2-positive expressing breast cancer cells. Using $^{64}$Cu-MM-302, the authors performed PET imaging in 19 patients with metastatic breast cancer, aiming to establish a correlation between therapeutic efficacy and the amount of drug reaching the metastases (as measured by PET) [123]. Although the use of $^{64}$Cu limits the imaging time window to up to 24-48h, which is not ideal for long-circulating liposomes, the authors found the expected biodistribution of a liposomal nanomedicine of this type, with long circulation and main accumulation in the liver and

spleen. Furthermore, in agreement with the study from Harrington *et al.* discussed above [16], they found significant liposomal uptake in primary tumors and metastases, as well as heterogeneity between subjects and lesions within the same subjects (Fig. 11A-B). Although the tumor uptake of MM-302 is likely to be affected by its HER2-targeted ligand, it is interesting to note that this heterogeneity was still observed. Finally, despite the low number of patients, an encouraging trend supporting a correlation between tumor uptake of MM-302 and the patient's disease progression-free survival, was observed (Fig. 11C).

MRI, despite its more challenging quantification and whole-body imaging capabilities, has also been used in a pilot clinical study to provide potential pre-therapeutic information of the likeliness of success for a nanomedicine treatment [124]. In this case, instead of imaging the actual therapeutic nanomedicine , MM-398 (Onivyde®) -a liposomal formulation of the topoisomerase I inhibitor irinotecan-, the authors used Ferumoxytol, a macrophage-avid, iron oxide, 30 nm-diameter nanoparticle clinically approved for treatment of iron-deficiency anemia. The hypothesis was that using Ferumoxytol as MRI contrast agent is likely to result in EPR-mediated tumor deposition and tumor-associated macrophage (TAM) uptake, providing a non-invasive MRI biomarker for MM-398 treatment. Using quantitative T2* methods, the authors evaluated this approach in 15 patients with confirmed solid tumors lesions (breast, cervical, head and neck, ovarian, pancreatic, and others). Their findings further confirmed marked heterogeneity in the uptake of Ferumoxytol in the different lesions, as well as an association between high levels of Ferumoxytol signal and reduction in tumor size after MM-398 treatment. Altogether, this study supports the use of quantitative Ferumoxytol-MRI as a potential imaging biomarker to predict EPR and nanomedicine therapeutic effects in patients, with the major advantage of relying on an already clinically approved agent [125].

## 8. Clinical translation and optimization of the use of nanomedicines

Successful translation of a nanomedicine requires properly designed clinical studies along the standard course of phase 1, 2, and 3 studies to demonstrate a significant added value of the new technology over the standard of care, either reduced toxicity or improved efficacy. While toxicity buffering is an important factor, a net gain in efficacy over conventional drugs will be the critical factor for successful translation of complex products such as nanomedicines in the foreseeable future. In this section, we focus on particular aspects of clinical testing that could provide a rapid insight on the performance of a newly developed nanomedicine and facilitate clinical translation.

**Phase 0 studies (microdosing)** [126]: Some of the critical issues in clinical drug development of nanomedicines are to ascertain formulation stability in circulation and the drug PK, and to induce a change in drug biodistribution. These aspects can be investigated using a very low dose or microdose by sensitive bioanalytical assays and by PET-CT imaging. The latter requires labeling of the nanomedicine with a PET radio-emitter but allows a rapid verification of the PK and tissue distribution of the new nanomedicine using a small GMP batch in a small group of patients with minimal regulatory barriers. The phase 0 study is a

powerful tool to evaluate nanomedicines with minimal patient exposure to the new agent and therefore minimal risk of toxicity. This early clinical feed-back may also allow adjustments and redesign of nanomedicines to obtain the desired *in vivo* properties in cancer patients, before embarking on an expensive path of clinical development. For example, the average therapeutic dose of PLD (Doxil) for a 70 kg-individual contains ~546 µmol of phospholipid, and a typical imaging dose for PET imaging with a long half-life tracer, such as $^{89}$Zr, is 75 MBq. According to Edmonds et al. [55], such an amount of $^{89}$Zr radioactivity can be loaded into a PLD dose of just 0.034 µmol of phospholipid, equivalen to 0.6% of the therapeutic dose and far below the accepted maximum value for microdosing (i.e., 1% of the therapeutic dose). Phase 0 studies may also serve as a preamble to Phase 1 studies as reported in a dog study with indocyanine-green entrapped in lipid nanoparticles, a product developed for surgical guidance in veterinary oncology [127]. Combined phase 0/phase 1 studies can be a helpful strategy to introduce nanomedicines safely to the pediatric population, a patient group which has been so far largely ignored in the clinical testing of nanomedicines. Microdosing can also help us to detect early in clinical development the phenomena of complement activation [128] whose clinical significance is unclear, and accelerated blood clearance [129] whose clinical occurrence has not been confirmed. Precisely, this last point is one of the problematic issues in microdosing testing of nanomedicines, including liposomes, which often have dose-dependent kinetics with more rapid clearance at low lipid doses. One way to tackle this problem is to administer with a co-dose of placebo nanoparticles as usually done in animal studies. Obviously, this approach may face a major regulatory hurdle since it is only feasible if the toxicity of the carrier nanoparticle is known and insignificant,

**Imaging the biodistribution of nanomedicines**: Imaging of nanomedicines in patients who are candidates for therapy or upon start of nanomedicine treatment, using a surrogate nanoparticle or a nanomedicine combining an imaging and a therapeutic agent (theranostics or nanotheranostics) will help evaluate the EPR of a particular tumor and a particular patient in real time as described in the previous section (Section 8: **Imaging and Theranostics**). This personalized approach, mostly using PET tracers or MRI contrast agents, can help select patients for treatment continuation based on the degree of EPR and detect potential tumor sites not effectively exposed to nanodrugs. Overall, it is likely to improve patient management by providing valuable information that can help redirect the treatment strategy and/or combine nanodrugs with additional tools (e.g. radiotherapy) to enhance the EPR effect (Fig. 12). Obviously, this approach is difficult to implement at the community clinic level and requires the infrastructure of comprehensive cancer centers.

**Pharmacokinetic and RES function monitoring**: The large clinical experience with PLD suggests that the PK of nanomedicines is affected by the clinical status of the cancer patient, by the RES functional activity, by concomitant drugs, and, probably also, by the presence of anti-PEG antibodies for pegylated nanoparticles. Assessment of pharmacokinetic parameters and of the functional activity of the RES may be a very useful tool to predict inter-patient and intra-patient course to course variability, pharmacokinetic interference of

concomitant drugs, toxicity, and even anti-tumor activity, as shown for the skin toxicity and Kaposi sarcoma response to PLD [130-132]. Since the plasma clearance of most nanomedicines is monoexponential with a small volume of distribution, it is possible to obtain an approximate PK evaluation with a minimal number of samples. For PLD, we have shown that two blood samples (1 hour post-infusion and 1 week post-infusion) provide an adequate evaluation of the clearance rate and of the risk of skin toxicity, thus providing a simplified approach to PK monitoring feasible in the routine clinical setting [130]. This approach is likely to be applicable to most nanomedicines. Patients with low Cmax and fast clearance of the nanodrug are likely to have suboptimal tumor drug delivery. Patients with slower drug clearance upon successive courses of treatment (due to impairment of RES function) are likely to develop toxicity. These PK warning signs may help clinicians in patient management.

Evaluating RES function is more complex and requires special probes. The blood clearance of $^{99m}$Tc -sulfur colloid (used for liver-spleen scintigraphy) is linked to the hepatosplenic RES activity and has been found to correlate with the PK of PLD in a human study [133]. The use of this marker could be used as a predictor of PLD clearance and help treatment decision. Other probes of phagocytic cell function based on blood monocytes and dendritic cells appear to provide as well useful information on the PK of nanodrugs based on preclinical and clinical studies [85, 134].

**Early intervention**: Nanomedicines have so far been tested mostly in advanced stages when cancers have already metastasized and developed multidrug resistance. Metastases often have fewer inflammatory cells and appear to have a weaker EPR effect than the primary tumor site [121]. Furthermore, tumor bulk is usually smaller in earlier than late stages and, while human data are still scarce, based on the preclinical data (see section 4, Fig. 6C-D), EPR is more effective in smaller tumors. Thus, the therapeutic potential of nanomedicines is probably greater treating the primary tumor site than the metastatic disease. Neoadjuvant or primary chemotherapy of cancer is being increasingly used prior to definitive surgery or radiotherapy and is the best setting to test the real added value of nanomedicine, once phase 1 studies have established the recommended safe dose. The impact may be substantial (increase of cure rate) rather than palliative (prolongation or improvement of quality of life). Unlike adjuvant post-surgical therapy, the readout of neoadjuvant therapy is quick since the regression of the tumor can be accurately documented by surgery. A few small studies with PLD in the neoadjuvant setting of breast cancer have been published (reviewed in [10]), but randomized phase 3 studies have not been launched. Hopefully, this will change in the coming years and some nanomedicines will be tested as add-on to standard therapies in the neoadjuvant setting.

**Multimodality and Combination therapy**: When possible, testing nanomedicines should be done early in clinical development in combination with other conventional anticancer drugs and, in specific cases, with other treatment modalities. This approach will help to pick up synergistic effects leading to convincing results where single therapy would have failed (e.g., approval of Onivyde in combination with 5FU, [135]). Of particular interest is the combination of nanomedicines with immune check point inhibitors, particularly since

nanoparticle formulations of cytotoxic drugs tend to be less toxic to the bone marrow than free drugs. An experimental therapeutic study in a mouse tumor model has shown that the combination of PLD and immune checkpoint inhibiting antibodies is extremely effective, far beyond than the activity of chemotherapy or immunotherapy alone [136]. Multimodality treatment using radiotherapy and other physical tools for regional or local cancer therapy is also an attractive option. Irradiation of tumors increases the influx of macrophages and tumor uptake of nanoparticles [31]. RFA generates an area of increase nanoparticle uptake in the rim of the heated area [137]. Hyperthermia increases the deposition of liposomes in tumors probably by increasing blood flow and vascular permeability [138]. Clearly, there is plenty of potential for combining nanomedicines with other modalities and drugs to improve outcomes.

**Co-encapsulation and co-delivery**: Co-encapsulation in a stable nano-formulation of two active agents preferably with non-overlapping toxicities and synergistic effects is a unique advantage of nanomedicines. By space and time co-delivery of two drugs with otherwise different PK-biodistribution profiles, we can exploit combination therapy at its best and achieve optimal synergistic activity. An example is a liposome-based formulation of cytarabine and daunorubicin at an optimized 5:1 drug-to-drug ratio, known as Vyxeos, approved for treatment of adult AML [139]. In this formulation the liposome carrier controls and nearly equalizes the PK of both drugs (Fig. 3) [140]. There are other examples of co-encapsulated drugs in liposome and polymeric formulations with positive results in animal models [141, 142]. While this approach is pharmaceutically and regulatory-wise challenging, it beholds promise for nanomedicine.

## 9. Future landscape of nanomedicines in cancer:

Nanomedicine is a promising tool with great potential for cancer therapy, but it is still an unregulated and heterogeneous bag of diverse products, some of which may not be pharmaceutically viable. Understanding and exploiting the interactions with the immune system, applying the insights from PK and imaging studies to improve safety and predict efficacy, and integrating nanomedicines with other therapeutic tools will help bring nanomedicine to the forefront of oncology and in early phase of cancer treatment.

Looking ahead at the future of medical innovations, priorities may shift towards approaches that are more widely applicable and sustainable, rather than personalized, highly resource-demanding and time intensive. This is especially relevant if medicines need to be deployed rapidly and globally to large portions of the population. As we endeavor to find ways to deliver cancer therapy with a high level of safety and efficacy based on sound pharmacological principles to an increasingly large and older population projected to develop cancer, the call for lessening the medical burden and number of hospital visits, and the associated medical costs will open new opportunities for sustainable and safe medical technologies. Nanomedicine, while pharmaceutically complex, is a mid-range cost and

sustainable technology with a generally improved safety profile that should gain a strong foothold and play an increasingly important role in cancer medicine.

**Figure Legends:**

**Fig. 1:** *In Vivo* Delivery of Nanomedicines – The route of a nanodrug: Extravasation of circulating liposomes into the tumor interstitial space and gradual drug release drug with free diffusion into cells. Uptake of liposomes by tumor-associated macrophages (TAM), and less so by tumor cells (TC). Trafficking of liposomes by way of endosomes and lysosomes, followed by drug release in cytosol, and crossing of the nucleo-cytoplasmic membrane with possible DNA damage.

**Fig. 2:** Blood Vessels - The Achilles Heel of Cancer. A: Normal tissue; B: Tumor tissue. From: Trédan et al., 2007 [14].

**Fig. 3:** (A) Whole-body gamma scintigraphy after injection of [DTPA-111In] Stealth PEGylated liposomes in a Kaposi Sarcoma patient, demonstrating high EPR-driven tumor uptake. Note the blood pool liposome image at 4 and 24h, fading only at 48h after injection. Together with the RES uptake, the tumor lesions, such as the one marked in left leg, show heavy deposition of liposomes, (B) Drug levels in tumor lesions of Kaposi sarcoma patients (n=13), 72h after treatment with free doxorubicin or PLD. The average tumor drug levels after PLD are 11-fold greater than after free drug. (C) Drug levels in adjacent normal skin and tumor lesions of Kaposi sarcoma patients (n=16), 48h after treatment with PLD. The average levels of PLD in tumor are 16-fold greater than in skin. Adapted from [16] and [25].

**Fig. 4:** A simplified flow chart for designing a nanoparticle-based cancer nanomedicine.

**Fig. 5:** Classification system for characterization of liposome drug products. Classification system for characterization of liposome drug products. Liposomes and other nanocarriers can be classified into 4 categories based on the drug release rate in circulation and the clearance by the reticulo-endothelial system (RES). Class IV are the most attractive nanocarriers since they are highly stable and have low affinity for the RES. Among the clinically approved nanomedicines, PLD, Myocet, and Daunoxome can be considered as examples of Class II, III, and IV respectively. From: Hsu & Huang, 2014 [46].

**Fig. 6:** Major pharmacological advantages of long-circulating (Stealth) nanodrug delivery systems: Reduced levels in heart muscle and Increased levels in tumor with dose dependence, and tumor size dependence. (A) Reduction of heart tissue uptake with liposomal doxorubicin (Dox) treatment. Peak tissue concentration and AUC are ~ 4-fold greater with free Dox than with liposomal Dox. BALB/c mice injected i.v. with 10 mg/kg Dox in free or liposomal form. (B) Effect of dose on peak tumor drug concentration of PLD and free doxorubicin. BALB/c mice with s.c. implants of M109 tumor injected i.v. with various doses of free doxorubicin or PLD and sacrificed 3 h (free doxorubicin) or 48 h (PLD) later. The gap between doxorubicin and PLD increases with dose. (C) Inverse correlation between tumor weight and liposome tumor uptake in Nude mice bearing s.c. implants of human KB tumor (n=62) injected i.v. with $^{111}$In-labeled liposomes 24 h before measurement. Spearman r = -0.573 , p<0.001. (D) Reduced liposome uptake in larger tumors grouped by cancer type in patients (n=15) injected with $^{111}$In-labeled PLD-like, drug free, liposomes. Adapted from: Tahover et al., 2014 [48]; Gabizon et al., 2002 [143]; Harrington et al., 2000 and 2001 [16, 62].

**Fig. 7:** Reduced systemic exposure (A), increased tumor cell targeting (B), and improved therapeutic activity (C) in an ascitic tumor model by intra-cavitary delivery of a folate-targeted nanomedicine. Folate Targeting of liposomes to ascitic tumor cells (J6456-FR lymphoma) in BALB/c mice. Plasma and ascites obtained 16h after i.p. injection of non-targeted (PLD) and folate-targeted liposomes (FT-PLD). Dose: 10mg/kg liposomal doxorubicin, 7 days post-tumor inoculation. From, Shmeeda et al. 2006 [82], and Gabizon et al. [83].

**Fig. 8:** *In vivo* interactions between nanomedicines and the immune system. A) Intravenously administered nanomedicines interact first with circulating immune proteins, primarily complement proteins and immunoglobulins, leading to nanoparticle opsonization and activation of the complement cascade, resulting in acute infusion reactions. Theoretically, interactions with circulating monocytes are also possible but the extent to which this occurs in vivo is unknown. B) The EPR effect is the primary determinant of nanomedicine accumulation in tumor tissue. Within the tumor stroma, nanoparticles are sequestered in tumor-associated macrophages (TAM) and myeloid-derived suppressor cells (MDSC) which act as drug reservoirs. Nanomedicines can also functionally polarize TAM, promoting an M1-like phenotype that has anti-tumoral activity. C) The RES is the primary mechanism of nanoparticle clearance from the circulation, nanoparticle breakdown, and metabolism of the payload (i.e., drug). Nanomedicines may also directly or indirectly interact with splenic lymphocytes, promoting cytokine production.

**Fig. 9:** PET imaging of radiolabelled PEGylated liposomes that encapsulate metal-chelating drugs. (A) Different radiometals can be incorporated into PEGylated liposomal alendronate (PLA) allowing the quantification of nanomedicine tumor accumulation in mouse models (10-15% ID/mL viable tumor volume in breast (MTLn3E, MDA-MB-231) and ovarian cancer tumors (SKOV3). (B) PET imaging using $^{89}$Zr-PLA shows high uptake (12-17% ID/g) in confirmed metastatic lymph nodes (LNmet) and lung metastases (Lumet) in the MTLn3E breast cancer model. Adapted from Edmonds et al. [55] and Man et al. [144].

**Fig. 10:** Preclinical studies show that liposome imaging can predict therapeutic efficacy. (**A, B**) Correlation between liposome accumulation in tumor assessed radiographically with an iodine contrast-loaded nanoliposome (A) and antitumor response to PLD (B). Tumors of treated group responded variably to PLD, as indicated by the individual tumor growth curves. Based on the deposition of X-ray contrast in tumor, animals with high tumor enhancement responded significantly better than those with poor tumor enhancement, $p < 0.003$. Arrow indicates day of PLD treatment. Untreated control data are presented as mean ± SD. Adapted from Kathanasis et al. [119]. **(C, D)** PET imaging of a PLD-like $^{89}$Zr-labelled PEGylated liposome predicts therapeutic efficacy of PLD in a 4T1 mouse model of breast cancer. **(C)** PET images of mice HD-10 (large tumour, high uptake), HD-07 (small tumor, high uptake) and HD-18 (medium-sized tumor, low uptake), demonstrating intertumoral uptake heterogeneity; **(D)** Tumour growth curves in cohorts with >25 mg/kg intratumoural DOX concentration (green), <25 mg/kg intratumoural Doxil concentration (red), and controls (black); tumours with higher doses of intratumoural doxorubicin (i.e. green group, measured with PET) had delayed tumor growth compared to the low uptake group (red) and control. Adapted from Perez-Medina et al. (2018) [145].

**Fig. 11:** Fig 11. (A) PET-CT imaging of $^{64}$Cu-MM-302 in breast cancer patients shows uptake in different lesions at several anatomical locations. Note that all lesions show positive (higher than background) PET signal, apart from the liver lesion that shows negative (lower than background) PET signal, which is likely the result of the lower concentration of phagocytic cells in tumors compared to liver tissue; (B) $^{64}$Cu-MM-302 lesion deposition data for different patients (lowest uptake lesion within each patient) in ascending order. Using ROC analysis, a deposition threshold was selected based on the inflection point of the graph. Patients to the right of the inflection point were designated as the "high uptake" group and those to the left as "low uptake". (B) Using the definitions described in (B), those patients in the "high uptake" group show a trend for longer PFS (progressive disease-free survival). Adapted from Lee et al., 2017 [56].

**Fig. 12:** Decision scheme supporting the design of a successful nanomedicine and selection of the optimal treatment depending on patient tumor physiological characteristics assessed by real time imaging. EPR = enhanced permeability and retention; IFP = interstitial fluid pressure. Adapted from Grodzinski et al. [6].

**Fig. 13:** Pharmacokinetics in human patients of liposome co-encapsulated cytarabine and daunorubicin (Vyxeos). Mean plasma cytarabine, daunorubicin, and metabolites (n=13) after 90-minute infusion. Note that both drugs reach a high Cmax and are cleared slowly and mono-exponentially at a nearly equal rate for the first 8 hours after infusion. The median 5-day half-lives are 31 and 22 hours for cytarabine and daunorubicin respectively [140]. This is in contrast to ~5-15-fold lower Cmax and t½ values of 3.8 h and 11.0 h for free cytarabine and free daunorubicin respectively [146]. Metabolites of both drugs behave different kinetically as expected from a drug-specific metabolic process post-liposome release. From Feldman et al., 2011 [140].

## References

[1] D. Hanahan, R.A. Weinberg, Hallmarks of cancer: the next generation, Cell, 144 (2011) 646-674.
[2] N.M. La-Beck, D.T. Nguyen, A.D. Le, S.K. Alzghari, S.T. Trinh, Optimizing Patient Outcomes with PD-1/PD-L1 Immune Checkpoint Inhibitors for the First-Line Treatment of Advanced Non-Small Cell Lung Cancer, Pharmacotherapy, (2020).
[3] N.M. La-Beck, G.W. Jean, C. Huynh, S.K. Alzghari, D.B. Lowe, Immune Checkpoint Inhibitors: New Insights and Current Place in Cancer Therapy, Pharmacotherapy, 35 (2015) 963-976.
[4] Y. Liu, B.M. Crawford, T. Vo-Dinh, Gold nanoparticles-mediated photothermal therapy and immunotherapy, Immunotherapy, 10 (2018) 1175-1188.
[5] P. Ma, R.J. Mumper, Paclitaxel Nano-Delivery Systems: A Comprehensive Review, J Nanomed Nanotechnol, 4 (2013) 1000164.
[6] P. Grodzinski, M. Kircher, M. Goldberg, A. Gabizon, Integrating Nanotechnology into Cancer Care, ACS Nano, 13 (2019) 7370-7376.
[7] G.H. Petersen, S.K. Alzghari, W. Chee, S.S. Sankari, N.M. La-Beck, Meta-analysis of clinical and preclinical studies comparing the anticancer efficacy of liposomal versus conventional non-liposomal doxorubicin, Journal of controlled release : official journal of the Controlled Release Society, 232 (2016) 255-264.
[8] R. Duncan, R. Gaspar, Nanomedicine(s) under the microscope, Molecular pharmaceutics, 8 (2011) 2101-2141.
[9] Y. Barenholz, Doxil(R)--the first FDA-approved nano-drug: lessons learned, J Control Release, 160 (2012) 117-134.
[10] A.A. Gabizon, Y. Patil, N.M. La-Beck, New insights and evolving role of pegylated liposomal doxorubicin in cancer therapy, Drug Resist Updat, 29 (2016) 90-106.
[11] D.A. Yardley, nab-Paclitaxel mechanisms of action and delivery, J Control Release, 170 (2013) 365-372.
[12] S.K. Golombek, J.N. May, B. Theek, L. Appold, N. Drude, F. Kiessling, T. Lammers, Tumor targeting via EPR: Strategies to enhance patient responses, Adv Drug Deliv Rev, 130 (2018) 17-38.
[13] H. Maeda, H. Nakamura, J. Fang, The EPR effect for macromolecular drug delivery to solid tumors: Improvement of tumor uptake, lowering of systemic toxicity, and distinct tumor imaging in vivo, Adv Drug Deliv Rev, 65 (2013) 71-79.
[14] O. Tredan, C.M. Galmarini, K. Patel, I.F. Tannock, Drug resistance and the solid tumor microenvironment, J Natl Cancer Inst, 99 (2007) 1441-1454.
[15] F. Man, T. Lammers, T.M.d.R.R, Imaging Nanomedicine-Based Drug Delivery: a Review of Clinical Studies, Mol Imaging Biol, 20 (2018) 683-695.
[16] K.J. Harrington, S. Mohammadtaghi, P.S. Uster, D. Glass, A.M. Peters, R.G. Vile, J.S. Stewart, Effective targeting of solid tumors in patients with locally advanced cancers by radiolabeled pegylated liposomes, Clinical cancer research : an official journal of the American Association for Cancer Research, 7 (2001) 243-254.
[17] T. Donnem, J. Hu, M. Ferguson, O. Adighibe, C. Snell, A.L. Harris, K.C. Gatter, F. Pezzella, Vessel co-option in primary human tumors and metastases: an obstacle to effective anti-angiogenic treatment?, Cancer medicine, 2 (2013) 427-436.
[18] Q. Guo, Z. Jin, Y. Yuan, R. Liu, T. Xu, H. Wei, X. Xu, S. He, S. Chen, Z. Shi, W. Hou, B. Hua, New Mechanisms of Tumor-Associated Macrophages on Promoting Tumor Progression: Recent Research Advances and Potential Targets for Tumor Immunotherapy, J Immunol Res, 2016 (2016) 9720912.
[19] S.K. Huang, K.D. Lee, K. Hong, D.S. Friend, D. Papahadjopoulos, Microscopic localization of sterically stabilized liposomes in colon carcinoma-bearing mice, Cancer Res, 52 (1992) 5135-5143.
[20] D.B. Kirpotin, D.C. Drummond, Y. Shao, M.R. Shalaby, K. Hong, U.B. Nielsen, J.D. Marks, C.C. Benz, J.W. Park, Antibody targeting of long-circulating lipidic nanoparticles does not increase tumor localization but does increase internalization in animal models, Cancer Res, 66 (2006) 6732-6740.

[21] H.Y. Kim, R. Li, T.S.C. Ng, G. Courties, C.B. Rodell, M. Prytyskach, R.H. Kohler, M.J. Pittet, M. Nahrendorf, R. Weissleder, M.A. Miller, Quantitative Imaging of Tumor-Associated Macrophages and Their Response to Therapy Using (64)Cu-Labeled Macrin, ACS Nano, 12 (2018) 12015-12029.
[22] R.K. Jain, Vascular and interstitial barriers to delivery of therapeutic agents in tumors, Cancer Metastasis Rev, 9 (1990) 253-266.
[23] T. Stylianopoulos, J.D. Martin, M. Snuderl, F. Mpekris, S.R. Jain, R.K. Jain, Coevolution of solid stress and interstitial fluid pressure in tumors during progression: implications for vascular collapse, Cancer Res, 73 (2013) 3833-3841.
[24] V.P. Chauhan, T. Stylianopoulos, J.D. Martin, Z. Popovic, O. Chen, W.S. Kamoun, M.G. Bawendi, D. Fukumura, R.K. Jain, Normalization of tumour blood vessels improves the delivery of nanomedicines in a size-dependent manner, Nat Nanotechnol, 7 (2012) 383-388.
[25] D.W. Northfelt, Pegylated liposomal doxorubicin (Doxil®) in the treatment of AIDS-related Kaposi's sarcoma, in: M.C. Woodle, G. Storm (Eds.) Long Circulating Liposomes: old drugs, new therapeutics, Springer-Verlag and Landes Bioscience, New York, 1998, pp. 127-145.
[26] A. Udhrain, K.M. Skubitz, D.W. Northfelt, Pegylated liposomal doxorubicin in the treatment of AIDS-related Kaposi's sarcoma, Int J Nanomedicine, 2 (2007) 345-352.
[27] F. Yuan, M. Leunig, S.K. Huang, D.A. Berk, D. Papahadjopoulos, R.K. Jain, Microvascular permeability and interstitial penetration of sterically stabilized (stealth) liposomes in a human tumor xenograft, Cancer Res, 54 (1994) 3352-3356.
[28] S. Sindhwani, A.M. Syed, J. Ngai, B.R. Kingston, L. Maiorino, J. Rothschild, P. MacMillan, Y. Zhang, N.U. Rajesh, T. Hoang, J.L.Y. Wu, S. Wilhelm, A. Zilman, S. Gadde, A. Sulaiman, B. Ouyang, Z. Lin, L. Wang, M. Egeblad, W.C.W. Chan, The entry of nanoparticles into solid tumours, Nat Mater, 19 (2020) 566-575.
[29] S.K. Huang, F.J. Martin, G. Jay, J. Vogel, D. Papahadjopoulos, D.S. Friend, Extravasation and transcytosis of liposomes in Kaposi's sarcoma-like dermal lesions of transgenic mice bearing the HIV tat gene, Am J Pathol, 143 (1993) 10-14.
[30] Y. Matsumoto, J.W. Nichols, K. Toh, T. Nomoto, H. Cabral, Y. Miura, R.J. Christie, N. Yamada, T. Ogura, M.R. Kano, Y. Matsumura, N. Nishiyama, T. Yamasoba, Y.H. Bae, K. Kataoka, Vascular bursts enhance permeability of tumour blood vessels and improve nanoparticle delivery, Nat Nanotechnol, 11 (2016) 533-538.
[31] M.A. Miller, R. Chandra, M.F. Cuccarese, C. Pfirschke, C. Engblom, S. Stapleton, U. Adhikary, R.H. Kohler, J.F. Mohan, M.J. Pittet, R. Weissleder, Radiation therapy primes tumors for nanotherapeutic delivery via macrophage-mediated vascular bursts, Sci Transl Med, 9 (2017).
[32] R. van der Meel, E. Sulheim, Y. Shi, F. Kiessling, W.J.M. Mulder, T. Lammers, Smart cancer nanomedicine, Nat Nanotechnol, 14 (2019) 1007-1017.
[33] F. Yuan, M. Dellian, D. Fukumura, M. Leunig, D.A. Berk, V.P. Torchilin, R.K. Jain, Vascular permeability in a human tumor xenograft: molecular size dependence and cutoff size, Cancer Res, 55 (1995) 3752-3756.
[34] K. Yokoi, D. Chan, M. Kojic, M. Milosevic, D. Engler, R. Matsunami, T. Tanei, Y. Saito, M. Ferrari, A. Ziemys, Liposomal doxorubicin extravasation controlled by phenotype-specific transport properties of tumor microenvironment and vascular barrier, J Control Release, 217 (2015) 293-299.
[35] K.J. Harrington, C.R. Lewanski, A.D. Northcote, J. Whittaker, H. Wellbank, R.G. Vile, A.M. Peters, J.S. Stewart, Phase I-II study of pegylated liposomal cisplatin (SPI-077) in patients with inoperable head and neck cancer, Annals of oncology : official journal of the European Society for Medical Oncology / ESMO, 12 (2001) 493-496.
[36] S. Bandak, D. Goren, A. Horowitz, D. Tzemach, A. Gabizon, Pharmacological studies of cisplatin encapsulated in long-circulating liposomes in mouse tumor models, Anti-cancer drugs, 10 (1999) 911-920.
[37] T. Daemen, G. Hofstede, M.T. Ten Kate, I.A. Bakker-Woudenberg, G.L. Scherphof, Liposomal doxorubicin-induced toxicity: depletion and impairment of phagocytic activity of liver macrophages, Int J Cancer, 61 (1995) 716-721.

[38] J. Choi, H.Y. Kim, E.J. Ju, J. Jung, J. Park, H.K. Chung, J.S. Lee, J.S. Lee, H.J. Park, S.Y. Song, S.Y. Jeong, E.K. Choi, Use of macrophages to deliver therapeutic and imaging contrast agents to tumors, Biomaterials, 33 (2012) 4195-4203.
[39] M.A. Miller, Y.R. Zheng, S. Gadde, C. Pfirschke, H. Zope, C. Engblom, R.H. Kohler, Y. Iwamoto, K.S. Yang, B. Askevold, N. Kolishetti, M. Pittet, S.J. Lippard, O.C. Farokhzad, R. Weissleder, Tumour-associated macrophages act as a slow-release reservoir of nano-therapeutic Pt(IV) pro-drug, Nat Commun, 6 (2015) 8692.
[40] S. Cecco, M. Aliberti, P. Baldo, E. Giacomin, R. Leone, Safety and efficacy evaluation of albumin-bound paclitaxel, Expert opinion on drug safety, 13 (2014) 511-520.
[41] M. Manzano, M. Vallet-Regi, Mesoporous silica nanoparticles in nanomedicine applications, J Mater Sci Mater Med, 29 (2018) 65.
[42] R. Mohammadpour, M. Yazdimamaghani, D.L. Cheney, J. Jedrzkiewicz, H. Ghandehari, Subchronic toxicity of silica nanoparticles as a function of size and porosity, J Control Release, 304 (2019) 216-232.
[43] F. Chen, K. Ma, B. Madajewski, L. Zhuang, L. Zhang, K. Rickert, M. Marelli, B. Yoo, M.Z. Turker, M. Overholtzer, T.P. Quinn, M. Gonen, P. Zanzonico, A. Tuesca, M.A. Bowen, L. Norton, J.A. Subramony, U. Wiesner, M.S. Bradbury, Ultrasmall targeted nanoparticles with engineered antibody fragments for imaging detection of HER2-overexpressing breast cancer, Nat Commun, 9 (2018) 4141.
[44] X. Zhang, F. Chen, M.Z. Turker, K. Ma, P. Zanzonico, F. Gallazzi, M.A. Shah, A.R. Prater, U. Wiesner, M.S. Bradbury, M.R. McDevitt, T.P. Quinn, Targeted melanoma radiotherapy using ultrasmall (177)Lu-labeled alpha-melanocyte stimulating hormone-functionalized core-shell silica nanoparticles, Biomaterials, 241 (2020) 119858.
[45] E. Christensen, J.R. Henriksen, J.T. Jorgensen, Y. Amitay, H. Shmeeda, A.A. Gabizon, A. Kjaer, T.L. Andresen, A.E. Hansen, Folate receptor targeting of radiolabeled liposomes reduces intratumoral liposome accumulation in human KB carcinoma xenografts, Int J Nanomedicine, 13 (2018) 7647-7656.
[46] L.F. Hsu, J.D. Huang, A statistical analysis to assess the most critical bioequivalence parameters for generic liposomal products, International journal of clinical pharmacology and therapeutics, 52 (2014) 1071-1082.
[47] A. Gabizon, H. Shmeeda, Y. Barenholz, Pharmacokinetics of pegylated liposomal Doxorubicin: review of animal and human studies, Clin Pharmacokinet, 42 (2003) 419-436.
[48] E. Tahover, Y.P. Patil, A.A. Gabizon, Emerging delivery systems to reduce doxorubicin cardiotoxicity and improve therapeutic index: focus on liposomes, Anticancer Drugs, 26 (2015) 241-258.
[49] A. Gabizon, D. Papahadjopoulos, Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors, Proc Natl Acad Sci U S A, 85 (1988) 6949-6953.
[50] D. Papahadjopoulos, T.M. Allen, A. Gabizon, E. Mayhew, K. Matthay, S.K. Huang, K.D. Lee, M.C. Woodle, D.D. Lasic, C. Redemann, et al., Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy, Proc Natl Acad Sci U S A, 88 (1991) 11460-11464.
[51] A.L. Klibanov, K. Maruyama, V.P. Torchilin, L. Huang, Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes, FEBS Lett, 268 (1990) 235-237.
[52] G. Haran, R. Cohen, L.K. Bar, Y. Barenholz, Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases, Biochim Biophys Acta, 1151 (1993) 201-215.
[53] A.A. Gabizon, Pegylated liposomal doxorubicin: metamorphosis of an old drug into a new form of chemotherapy, Cancer Invest, 19 (2001) 424-436.
[54] A. Pendlebury, R. DeBernardo, P.G. Rose, Long-term use of pegylated liposomal doxorubicin to a cumulative dose of 4600 mg/m2 in recurrent ovarian cancer, Anticancer Drugs, 28 (2017) 815-817.
[55] S. Edmonds, A. Volpe, H. Shmeeda, A.C. Parente-Pereira, R. Radia, J. Baguna-Torres, I. Szanda, G.W. Severin, L. Livieratos, P.J. Blower, J. Maher, G.O. Fruhwirth, A. Gabizon, T.M.d.R. R, Exploiting

the Metal-Chelating Properties of the Drug Cargo for In Vivo Positron Emission Tomography Imaging of Liposomal Nanomedicines, ACS Nano, 10 (2016) 10294-10307.
[56] H. Lee, A.F. Shields, B.A. Siegel, K.D. Miller, I. Krop, C.X. Ma, P.M. LoRusso, P.N. Munster, K. Campbell, D.F. Gaddy, S.C. Leonard, E. Geretti, S.J. Blocker, D.B. Kirpotin, V. Moyo, T.J. Wickham, B.S. Hendriks, (64)Cu-MM-302 Positron Emission Tomography Quantifies Variability of Enhanced Permeability and Retention of Nanoparticles in Relation to Treatment Response in Patients with Metastatic Breast Cancer, Clin Cancer Res, 23 (2017) 4190-4202.
[57] J. Cummings, C.S. McArdle, Studies on the in vivo disposition of adriamycin in human tumours which exhibit different responses to the drug, British journal of cancer, 53 (1986) 835-838.
[58] S. Stallard, J.G. Morrison, W.D. George, S.B. Kaye, Distribution of doxorubicin to normal breast and tumour tissue in patients undergoing mastectomy, Cancer Chemotherapy and Pharmacology, 25 (1990) 286-290.
[59] V. Troger, E. Francois, M. Frenay, M. Namer, G. Milano, Analysis of tissue platinum distribution in patients with cancer of the oesophagus, European Journal of Cancer and Clinical Oncology, 27 (1991) 256-259.
[60] Z. Symon, A. Peyser, D. Tzemach, O. Lyass, E. Sucher, E. Shezen, A. Gabizon, Selective delivery of doxorubicin to patients with breast carcinoma metastases by stealth liposomes, Cancer, 86 (1999) 72-78.
[61] A. Gabizon, D.C. Price, J. Huberty, R.S. Bresalier, D. Papahadjopoulos, Effect of liposome composition and other factors on the targeting of liposomes to experimental tumors: biodistribution and imaging studies, Cancer Res, 50 (1990) 6371-6378.
[62] K.J. Harrington, G. Rowlinson-Busza, K.N. Syrigos, R.M. Abra, P.S. Uster, A.M. Peters, J.S. Stewart, Influence of tumour size on uptake of (111)In-DTPA-labelled pegylated liposomes in a human tumour xenograft model, British journal of cancer, 83 (2000) 684-688.
[63] S. Wilhelm, A.J. Tavares, Q. Dai, S. Ohta, J. Audet, H.F. Dvorak, W.C.W. Chan, Analysis of nanoparticle delivery to tumours, Nature Reviews Materials, 1 (2016) 16014.
[64] Q. Hu, C.J. Rijcken, R. Bansal, W.E. Hennink, G. Storm, J. Prakash, Complete regression of breast tumour with a single dose of docetaxel-entrapped core-cross-linked polymeric micelles, Biomaterials, 53 (2015) 370-378.
[65] M. Talelli, M. Barz, C.J. Rijcken, F. Kiessling, W.E. Hennink, T. Lammers, Core-Crosslinked Polymeric Micelles: Principles, Preparation, Biomedical Applications and Clinical Translation, Nano Today, 10 (2015) 93-117.
[66] H. Cabral, M. Murakami, H. Hojo, Y. Terada, M.R. Kano, U.I. Chung, N. Nishiyama, K. Kataoka, Targeted therapy of spontaneous murine pancreatic tumors by polymeric micelles prolongs survival and prevents peritoneal metastasis, Proc Natl Acad Sci U S A, 110 (2013) 11397-11402.
[67] P.C. Lyon, M.D. Gray, C. Mannaris, L.K. Folkes, M. Stratford, L. Campo, D.Y.F. Chung, S. Scott, M. Anderson, R. Goldin, R. Carlisle, F. Wu, M.R. Middleton, F.V. Gleeson, C.C. Coussios, Safety and feasibility of ultrasound-triggered targeted drug delivery of doxorubicin from thermosensitive liposomes in liver tumours (TARDOX): a single-centre, open-label, phase 1 trial, The Lancet. Oncology, 19 (2018) 1027-1039.
[68] H.C. Besse, A.D. Barten-van Rijbroek, K.M.G. van der Wurff-Jacobs, C. Bos, C.T.W. Moonen, R. Deckers, Tumor Drug Distribution after Local Drug Delivery by Hyperthermia, In Vivo, Cancers (Basel), 11 (2019).
[69] B. Wang, C.V. Galliford, P.S. Low, Guiding principles in the design of ligand-targeted nanomedicines, Nanomedicine (London, England), 9 (2014) 313-330.
[70] P. Munster, I.E. Krop, P. LoRusso, C. Ma, B.A. Siegel, A.F. Shields, I. Molnar, T.J. Wickham, J. Reynolds, K. Campbell, B.S. Hendriks, B.S. Adiwijaya, E. Geretti, V. Moyo, K.D. Miller, Safety and pharmacokinetics of MM-302, a HER2-targeted antibody-liposomal doxorubicin conjugate, in patients with advanced HER2-positive breast cancer: a phase 1 dose-escalation study, British journal of cancer, 119 (2018) 1086-1093.

[71] D.D. Von Hoff, M.M. Mita, R.K. Ramanathan, G.J. Weiss, A.C. Mita, P.M. LoRusso, H.A. Burris, 3rd, L.L. Hart, S.C. Low, D.M. Parsons, S.E. Zale, J.M. Summa, H. Youssoufian, J.C. Sachdev, Phase I Study of PSMA-Targeted Docetaxel-Containing Nanoparticle BIND-014 in Patients with Advanced Solid Tumors, Clin Cancer Res, 22 (2016) 3157-3163.
[72] K. Miller, J. Cortes, S.A. Hurvitz, I.E. Krop, D. Tripathy, S. Verma, K. Riahi, J.G. Reynolds, T.J. Wickham, I. Molnar, D.A. Yardley, HERMIONE: a randomized Phase 2 trial of MM-302 plus trastuzumab versus chemotherapy of physician's choice plus trastuzumab in patients with previously treated, anthracycline-naive, HER2-positive, locally advanced/metastatic breast cancer, BMC cancer, 16 (2016) 352.
[73] A.F.e. al., A phase I dose-finding and pharmacokinetic study of CPC634 -nanoparticle entrapped docetaxel- in patients with advanced solid tumors

Proc. ASCO Annual Meeting, 2019, pp. Abst. ID 3026.
[74] P.S. Low, W.A. Henne, D.D. Doorneweerd, Discovery and development of folic-acid-based receptor targeting for imaging and therapy of cancer and inflammatory diseases, Acc Chem Res, 41 (2008) 120-129.
[75] Targeted Drug Strategies for Cancer and Inflammation, Springer US, New York, 2011.
[76] D. Goren, A.T. Horowitz, S. Zalipsky, M.C. Woodle, Y. Yarden, A. Gabizon, Targeting of stealth liposomes to erbB-2 (Her/2) receptor: in vitro and in vivo studies, Br J Cancer, 74 (1996) 1749-1756.
[77] A. Gabizon, H. Shmeeda, A.T. Horowitz, S. Zalipsky, Tumor cell targeting of liposome-entrapped drugs with phospholipid-anchored folic acid-PEG conjugates, Adv Drug Deliv Rev, 56 (2004) 1177-1192.
[78] A. Chaudhury, S. Das, R.M. Bunte, G.N. Chiu, Potent therapeutic activity of folate receptor-targeted liposomal carboplatin in the localized treatment of intraperitoneally grown human ovarian tumor xenograft, Int J Nanomedicine, 7 (2012) 739-751.
[79] S. Li, B. Goins, B.A. Hrycushko, W.T. Phillips, A. Bao, Feasibility of eradication of breast cancer cells remaining in postlumpectomy cavity and draining lymph nodes following intracavitary injection of radioactive immunoliposomes, Mol Pharm, 9 (2012) 2513-2522.
[80] A.A. Gabizon, H. Shmeeda, S. Zalipsky, Pros and cons of the liposome platform in cancer drug targeting, Journal of liposome research, 16 (2006) 175-183.
[81] Y. Patil, Y. Amitay, P. Ohana, H. Shmeeda, A. Gabizon, Targeting of pegylated liposomal mitomycin-C prodrug to the folate receptor of cancer cells: Intracellular activation and enhanced cytotoxicity, Journal of controlled release : official journal of the Controlled Release Society, 225 (2016) 87-95.
[82] H. Shmeeda, L. Mak, D. Tzemach, P. Astrahan, M. Tarshish, A. Gabizon, Intracellular uptake and intracavitary targeting of folate-conjugated liposomes in a mouse lymphoma model with up-regulated folate receptors, Mol Cancer Ther, 5 (2006) 818-824.
[83] A. Gabizon, D. Tzemach, J. Gorin, L. Mak, Y. Amitay, H. Shmeeda, S. Zalipsky, Improved therapeutic activity of folate-targeted liposomal doxorubicin in folate receptor-expressing tumor models, Cancer Chemother Pharmacol, 66 (2010) 43-52.
[84] M.A. Dobrovolskaia, P. Aggarwal, J.B. Hall, S.E. McNeil, Preclinical studies to understand nanoparticle interaction with the immune system and its potential effects on nanoparticle biodistribution, Molecular pharmaceutics, 5 (2008) 487-495.
[85] W.P. Caron, J.C. Lay, A.M. Fong, N.M. La-Beck, P. Kumar, S.E. Newman, H. Zhou, J.H. Monaco, D.L. Clarke-Pearson, W.R. Brewster, L. Van Le, V.L. Bae-Jump, P.A. Gehrig, W.C. Zamboni, Translational studies of phenotypic probes for the mononuclear phagocyte system and liposomal pharmacology, The Journal of pharmacology and experimental therapeutics, 347 (2013) 599-606.
[86] A.S. Fortuin, R. Bruggemann, J. van der Linden, I. Panfilov, B. Israel, T.W.J. Scheenen, J.O. Barentsz, Ultra-small superparamagnetic iron oxides for metastatic lymph node detection: back on the block, Wiley Interdiscip Rev Nanomed Nanobiotechnol, 10 (2018).

[87] A. Gabizon, D. Goren, Z. Fuks, Y. Barenholz, A. Dagan, A. Meshorer, Enhancement of adriamycin delivery to liver metastatic cells with increased tumoricidal effect using liposomes as drug carriers, Cancer Res, 43 (1983) 4730-4735.
[88] S.M. Zeisberger, B. Odermatt, C. Marty, A.H. Zehnder-Fjallman, K. Ballmer-Hofer, R.A. Schwendener, Clodronate-liposome-mediated depletion of tumour-associated macrophages: a new and highly effective antiangiogenic therapy approach, British journal of cancer, 95 (2006) 272-281.
[89] R. Rajan, M.K. Sabnani, V. Mavinkurve, H. Shmeeda, H. Mansouri, S. Bonkoungou, A.D. Le, L.M. Wood, A.A. Gabizon, N.M. La-Beck, Liposome-induced immunosuppression and tumor growth is mediated by macrophages and mitigated by liposome-encapsulated alendronate, Journal of controlled release : official journal of the Controlled Release Society, 271 (2018) 139-148.
[90] N.M. La-Beck, X. Liu, L.M. Wood, Harnessing Liposome Interactions With the Immune System for the Next Breakthrough in Cancer Drug Delivery, Front Pharmacol, 10 (2019) 220.
[91] R.C. Ji, Lymph Nodes and Cancer Metastasis: New Perspectives on the Role of Intranodal Lymphatic Sinuses, Int J Mol Sci, 18 (2016).
[92] G. Caracciolo, Liposome-protein corona in a physiological environment: challenges and opportunities for targeted delivery of nanomedicines, Nanomedicine, 11 (2015) 543-557.
[93] C. Corbo, R. Molinaro, F. Taraballi, N.E. Toledano Furman, M.B. Sherman, A. Parodi, F. Salvatore, E. Tasciotti, Effects of the protein corona on liposome-liposome and liposome-cell interactions, Int J Nanomedicine, 11 (2016) 3049-3063.
[94] F. Barbero, L. Russo, M. Vitali, J. Piella, I. Salvo, M.L. Borrajo, M. Busquets-Fite, R. Grandori, N.G. Bastus, E. Casals, V. Puntes, Formation of the Protein Corona: The Interface between Nanoparticles and the Immune System, Semin Immunol, 34 (2017) 52-60.
[95] D.F. Nellis, S.L. Giardina, G.M. Janini, S.R. Shenoy, J.D. Marks, R. Tsai, D.C. Drummond, K. Hong, J.W. Park, T.F. Ouellette, S.C. Perkins, D.B. Kirpotin, Preclinical manufacture of anti-HER2 liposome-inserting, scFv-PEG-lipid conjugate. 2. Conjugate micelle identity, purity, stability, and potency analysis, Biotechnol Prog, 21 (2005) 221-232.
[96] J. Szebeni, F. Muggia, A. Gabizon, Y. Barenholz, Activation of complement by therapeutic liposomes and other lipid excipient-based therapeutic products: prediction and prevention, Advanced drug delivery reviews, 63 (2011) 1020-1030.
[97] S.M. Moghimi, J. Szebeni, Stealth liposomes and long circulating nanoparticles: critical issues in pharmacokinetics, opsonization and protein-binding properties, Prog Lipid Res, 42 (2003) 463-478.
[98] S.M. Moghimi, Cancer nanomedicine and the complement system activation paradigm: anaphylaxis and tumour growth, J Control Release, 190 (2014) 556-562.
[99] M.K. Sabnani, R. Rajan, B. Rowland, V. Mavinkurve, L.M. Wood, A.A. Gabizon, N.M. La-Beck, Liposome promotion of tumor growth is associated with angiogenesis and inhibition of antitumor immune responses, Nanomedicine, 11 (2015) 259-262.
[100] S.M. Moghimi, Z.S. Farhangrazi, Just so stories: the random acts of anti-cancer nanomedicine performance, Nanomedicine, 10 (2014) 1661-1666.
[101] Q. Yang, T.M. Jacobs, J.D. McCallen, D.T. Moore, J.T. Huckaby, J.N. Edelstein, S.K. Lai, Analysis of Pre-existing IgG and IgM Antibodies against Polyethylene Glycol (PEG) in the General Population, Anal Chem, 88 (2016) 11804-11812.
[102] T. Lammers, L.Y. Rizzo, G. Storm, F. Kiessling, Personalized nanomedicine, Clin Cancer Res, 18 (2012) 4889-4894.
[103] B. Theek, L.Y. Rizzo, J. Ehling, F. Kiessling, T. Lammers, The Theranostic Path to Personalized Nanomedicine, Clin Transl Imaging, 2 (2014) 66-76.
[104] I.S. Vizirianakis, D.G. Fatouros, Personalized nanomedicine: paving the way to the practical clinical utility of genomics and nanotechnology advancements, Adv Drug Deliv Rev, 64 (2012) 1359-1362.
[105] R.D. Badawi, H. Shi, P. Hu, S. Chen, T. Xu, P.M. Price, Y. Ding, B.A. Spencer, L. Nardo, W. Liu, J. Bao, T. Jones, H. Li, S.R. Cherry, First Human Imaging Studies with the EXPLORER Total-Body PET Scanner, J Nucl Med, 60 (2019) 299-303.

[106] F. Man, P.J. Gawne, T.M.d.R. R, Nuclear imaging of liposomal drug delivery systems: A critical review of radiolabelling methods and applications in nanomedicine, Adv Drug Deliv Rev, 143 (2019) 134-160.
[107] T. van der Geest, P. Laverman, D. Gerrits, G.M. Franssen, J.M. Metselaar, G. Storm, O.C. Boerman, Comparison of three remote radiolabelling methods for long-circulating liposomes, J Control Release, 220 (2015) 239-244.
[108] K.J. Hwang, Modes of interaction of (In3+)-8-hydroxyquinoline with membrane bilayer, J Nucl Med, 19 (1978) 1162-1170.
[109] O.C. Boerman, G. Storm, W.J. Oyen, L. van Bloois, J.W. van der Meer, R.A. Claessens, D.J. Crommelin, F.H. Corstens, Sterically stabilized liposomes labeled with indium-111 to image focal infection, J Nucl Med, 36 (1995) 1639-1644.
[110] K.J. Harrington, G. Rowlinson-Busza, K.N. Syrigos, P.S. Uster, R.M. Abra, J.S.W. Stewart, Biodistribution and pharmacokinetics of 111In-DTPA-labelled pegylated liposomes in a human tumour xenograft model: implications for novel targeting strategies, British Journal of Cancer, 83 (2000) 232-238.
[111] A. Gabizon, J. Hliberty, R.M. Straubinger, D.C. Price, D. Papahadjopoulos, An Improved Method for in Vivo Tracing and Imaging of Liposomes Using a Gallium 67 Deferoxamine Complex, Journal of Liposome Research, 1 (2008) 123-135.
[112] A.L. Petersen, T. Binderup, P. Rasmussen, J.R. Henriksen, D.R. Elema, A. Kjær, T.L. Andresen, 64Cu loaded liposomes as positron emission tomography imaging agents, Biomaterials, 32 (2011) 2334-2341.
[113] S. Edmonds, A. Volpe, H. Shmeeda, A.C. Parente-Pereira, R. Radia, J. Baguña-Torres, I. Szanda, G.W. Severin, L. Livieratos, P.J. Blower, J. Maher, G.O. Fruhwirth, A. Gabizon, R. T. M. de Rosales, Exploiting the Metal-Chelating Properties of the Drug Cargo for In Vivo Positron Emission Tomography Imaging of Liposomal Nanomedicines, ACS Nano, 10 (2016) 10294-10307.
[114] A.I. Jensen, G.W. Severin, A.E. Hansen, F.F. Fliedner, R. Eliasen, L. Parhamifar, A. Kjær, T.L. Andresen, J.R. Henriksen, Remote-loading of liposomes with manganese-52 and in vivo evaluation of the stabilities of 52Mn-DOTA and 64Cu-DOTA using radiolabelled liposomes and PET imaging, Journal of Controlled Release, 269 (2018) 100-109.
[115] P. Gawne, F. Man, J. Fonslet, P. Radia, J. Bordoloi, M. Cleveland, P. Jimenez-Royo, A. Gabizon, P.J. Blower, N. Long, R.T.M. de Rosales, Manganese-52: applications in cell radiolabelling and liposomal nanomedicine PET imaging using oxine (8-hydroxyquinoline) as an ionophore, Dalton Transactions, 47 (2018) 9283-9293.
[116] F. Man, L. Lim, A. Volpe, A. Gabizon, H. Shmeeda, B. Draper, A.C. Parente-Pereira, J. Maher, P.J. Blower, G.O. Fruhwirth, R. T.M. de Rosales, In Vivo PET Tracking of 89Zr-Labeled Vγ9Vδ2 T Cells to Mouse Xenograft Breast Tumors Activated with Liposomal Alendronate, Molecular Therapy, 27 (2019) 219-229.
[117] P.J. Gawne, F. Clarke, K. Turjeman, A.P. Cope, N.J. Long, Y. Barenholz, S.Y.A. Terry, R.T.M. de Rosales, PET Imaging of Liposomal Glucocorticoids using <sup>89</sup>Zr-oxine: Theranostic Applications in Inflammatory Arthritis, Theranostics, 10 (2020) 3867-3879.
[118] C. Perez-Medina, D. Abdel-Atti, J. Tang, Y. Zhao, Z.A. Fayad, J.S. Lewis, W.J.M. Mulder, T. Reiner, Nanoreporter PET predicts the efficacy of anti-cancer nanotherapy, Nat Commun, 7 (2016) 11838.
[119] E. Karathanasis, S. Suryanarayanan, S.R. Balusu, K. McNeeley, I. Sechopoulos, A. Karellas, A.V. Annapragada, R.V. Bellamkonda, Imaging nanoprobe for prediction of outcome of nanoparticle chemotherapy by using mammography, Radiology, 250 (2009) 398-406.
[120] F. Danhier, To exploit the tumor microenvironment: Since the EPR effect fails in the clinic, what is the future of nanomedicine?, Journal of Controlled Release, 244 (2016) 108-121.
[121] U. Prabhakar, H. Maeda, R.K. Jain, E.M. Sevick-Muraca, W. Zamboni, O.C. Farokhzad, S.T. Barry, A. Gabizon, P. Grodzinski, D.C. Blakey, Challenges and key considerations of the enhanced

permeability and retention effect for nanomedicine drug delivery in oncology, Cancer Res, 73 (2013) 2412-2417.
[122] H. Lee, J. Zheng, D. Gaddy, K.D. Orcutt, S. Leonard, E. Geretti, J. Hesterman, C. Harwell, J. Hoppin, D.A. Jaffray, T. Wickham, B.S. Hendriks, D. Kirpotin, A gradient-loadable 64Cu-chelator for quantifying tumor deposition kinetics of nanoliposomal therapeutics by positron emission tomography, Nanomedicine: Nanotechnology, Biology and Medicine, 11 (2015) 155-165.
[123] H. Lee, A.F. Shields, B.A. Siegel, K.D. Miller, I. Krop, C.X. Ma, P.M. LoRusso, P.N. Munster, K. Campbell, D.F. Gaddy, S.C. Leonard, E. Geretti, S.J. Blocker, D.B. Kirpotin, V. Moyo, T.J. Wickham, B.S. Hendriks, 64Cu-MM-302 Positron Emission Tomography Quantifies Variability of Enhanced Permeability and Retention of Nanoparticles in Relation to Treatment Response in Patients with Metastatic Breast Cancer, Clinical Cancer Research, 23 (2017) 4190-4202.
[124] R.K. Ramanathan, R.L. Korn, N. Raghunand, J.C. Sachdev, R.G. Newbold, G. Jameson, G.J. Fetterly, J. Prey, S.G. Klinz, J. Kim, J. Cain, B.S. Hendriks, D.C. Drummond, E. Bayever, J.B. Fitzgerald, Correlation between Ferumoxytol Uptake in Tumor Lesions by MRI and Response to Nanoliposomal Irinotecan in Patients with Advanced Solid Tumors: A Pilot Study, Clinical Cancer Research, 23 (2017) 3638-3648.
[125] M.A. Miller, S. Arlauckas, R. Weissleder, Prediction of Anticancer Nanotherapy Efficacy by Imaging, Nanotheranostics, 1 (2017) 296-312.
[126] T. Burt, K. Yoshida, G. Lappin, L. Vuong, C. John, S.N. de Wildt, Y. Sugiyama, M. Rowland, Microdosing and Other Phase 0 Clinical Trials: Facilitating Translation in Drug Development, Clin Transl Sci, 9 (2016) 74-88.
[127] D. Sayag, Q. Cabon, I. Texier, F.P. Navarro, R. Boisgard, D. Virieux-Watrelot, C. Carozzo, F. Ponce, Phase-0/phase-I study of dye-loaded lipid nanoparticles for near-infrared fluorescence imaging in healthy dogs, European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik e.V, 100 (2016) 85-93.
[128] J. Szebeni, G. Storm, Complement activation as a bioequivalence issue relevant to the development of generic liposomes and other nanoparticulate drugs, Biochem Biophys Res Commun, 468 (2015) 490-497.
[129] Y. Mima, Y. Hashimoto, T. Shimizu, H. Kiwada, T. Ishida, Anti-PEG IgM Is a Major Contributor to the Accelerated Blood Clearance of Polyethylene Glycol-Conjugated Protein, Molecular pharmaceutics, 12 (2015) 2429-2435.
[130] A. Gabizon, H. Shmeeda, T. Grenader, Pharmacological basis of pegylated liposomal doxorubicin: impact on cancer therapy, Eur J Pharm Sci, 45 (2012) 388-398.
[131] O. Lyass, B. Uziely, R. Ben-Yosef, D. Tzemach, N.I. Heshing, M. Lotem, G. Brufman, A. Gabizon, Correlation of toxicity with pharmacokinetics of pegylated liposomal doxorubicin (Doxil) in metastatic breast carcinoma, Cancer, 89 (2000) 1037-1047.
[132] M.A. Amantea, A. Forest, D.W. Northfelt, R. Mamelok, Population pharmacokinetics and pharmacodynamics of pegylated-liposomal doxorubicin in patients with AIDS-related Kaposi's sarcoma, Clin Pharmacol Ther, 61 (1997) 301-311.
[133] H. Giovinazzo, P. Kumar, A. Sheikh, K.M. Brooks, M. Ivanovic, M. Walsh, W.P. Caron, R.J. Kowalsky, G. Song, A. Whitlow, D.L. Clarke-Pearson, W.R. Brewster, L. Van Le, B.A. Zamboni, V. Bae-Jump, P.A. Gehrig, W.C. Zamboni, Technetium Tc 99m sulfur colloid phenotypic probe for the pharmacokinetics and pharmacodynamics of PEGylated liposomal doxorubicin in women with ovarian cancer, Cancer Chemother Pharmacol, 77 (2016) 565-573.
[134] N.M. La-Beck, B.A. Zamboni, A. Gabizon, H. Schmeeda, M. Amantea, P.A. Gehrig, W.C. Zamboni, Factors affecting the pharmacokinetics of pegylated liposomal doxorubicin in patients, Cancer Chemother Pharmacol, 69 (2012) 43-50.
[135] A. Wang-Gillam, C.P. Li, G. Bodoky, A. Dean, Y.S. Shan, G. Jameson, T. Macarulla, K.H. Lee, D. Cunningham, J.F. Blanc, R.A. Hubner, C.F. Chiu, G. Schwartsmann, J.T. Siveke, F. Braiteh, V. Moyo, B. Belanger, N. Dhindsa, E. Bayever, D.D. Von Hoff, L.T. Chen, N.-S. Group, Nanoliposomal irinotecan

with fluorouracil and folinic acid in metastatic pancreatic cancer after previous gemcitabine-based therapy (NAPOLI-1): a global, randomised, open-label, phase 3 trial, Lancet, 387 (2016) 545-557.
[136] J. Rios-Doria, N. Durham, L. Wetzel, R. Rothstein, J. Chesebrough, N. Holoweckyj, W. Zhao, C.C. Leow, R. Hollingsworth, Doxil synergizes with cancer immunotherapies to enhance antitumor responses in syngeneic mouse models, Neoplasia (New York, N.Y.), 17 (2015) 661-670.
[137] W.L. Monsky, J.B. Kruskal, A.N. Lukyanov, G.D. Girnun, M. Ahmed, G.S. Gazelle, J.C. Huertas, K.E. Stuart, V.P. Torchilin, S.N. Goldberg, Radio-frequency ablation increases intratumoral liposomal doxorubicin accumulation in a rat breast tumor model, Radiology, 224 (2002) 823-829.
[138] S.K. Huang, P.R. Stauffer, K. Hong, J.W. Guo, T.L. Phillips, A. Huang, D. Papahadjopoulos, Liposomes and hyperthermia in mice: increased tumor uptake and therapeutic efficacy of doxorubicin in sterically stabilized liposomes, Cancer Res, 54 (1994) 2186-2191.
[139] M. Alfayez, H. Kantarjian, T. Kadia, F. Ravandi-Kashani, N. Daver, CPX-351 (vyxeos) in AML, Leukemia & lymphoma, 61 (2020) 288-297.
[140] E.J. Feldman, J.E. Lancet, J.E. Kolitz, E.K. Ritchie, G.J. Roboz, A.F. List, S.L. Allen, E. Asatiani, L.D. Mayer, C. Swenson, A.C. Louie, First-in-man study of CPX-351: a liposomal carrier containing cytarabine and daunorubicin in a fixed 5:1 molar ratio for the treatment of relapsed and refractory acute myeloid leukemia, J Clin Oncol, 29 (2011) 979-985.
[141] H. Shmeeda, Y. Amitay, J. Gorin, D. Tzemach, L. Mak, S.T. Stern, Y. Barenholz, A. Gabizon, Coencapsulation of alendronate and doxorubicin in pegylated liposomes: a novel formulation for chemoimmunotherapy of cancer, J Drug Target, (2016) 1-12.
[142] R.X. Zhang, P. Cai, T. Zhang, K. Chen, J. Li, J. Cheng, K.S. Pang, H.A. Adissu, A.M. Rauth, X.Y. Wu, Polymer-lipid hybrid nanoparticles synchronize pharmacokinetics of co-encapsulated doxorubicin-mitomycin C and enable their spatiotemporal co-delivery and local bioavailability in breast tumor, Nanomedicine : nanotechnology, biology, and medicine, 12 (2016) 1279-1290.
[143] A. Gabizon, D. Tzemach, L. Mak, M. Bronshtein, A.T. Horowitz, Dose dependency of pharmacokinetics and therapeutic efficacy of pegylated liposomal doxorubicin (DOXIL) in murine models, J Drug Target, 10 (2002) 539-548.
[144] F. Man, L. Lim, A. Volpe, A. Gabizon, H. Shmeeda, B. Draper, A.C. Parente-Pereira, J. Maher, P.J. Blower, G.O. Fruhwirth, T.M.d.R. R, In Vivo PET Tracking of (89)Zr-Labeled Vgamma9Vdelta2 T Cells to Mouse Xenograft Breast Tumors Activated with Liposomal Alendronate, Mol Ther, 27 (2019) 219-229.
[145] C. Perez-Medina, D. Abdel-Atti, J. Tang, Y. Zhao, Z.A. Fayad, J.S. Lewis, W.J. Mulder, T. Reiner, Nanoreporter PET predicts the efficacy of anti-cancer nanotherapy, Nat Commun, 7 (2016) 11838.
[146] D.R. Liston, M. Davis, Clinically Relevant Concentrations of Anticancer Drugs: A Guide for Nonclinical Studies, Clin Cancer Res, 23 (2017) 3489-3498.

Table 1: Nanoparticle-based products for cancer approved by FDA and/or EMA

| **Product** | **Indication in cancer** |
|---|---|
| Pegylated Liposomal Doxorubicin (Doxil, generics) | Kaposi Sarcoma, Ovary, Breast, Myeloma |
| Liposomal Daunorubicin (DaunoXome) | Kaposi Sarcoma |
| NAB-Paclitaxel (Abraxane)* | Breast, Lung, Pancreas |
| Liposomal Doxorubicin (Myocet) | Breast |
| Liposomal Vincristine (Marqibo) | Adult A.L.L. |
| Low-pegylated Liposomal Irinotecan (Onivyde) | Pancreas |
| Liposomal Cytarabine+Daunorubicin (Vyxeos) | Adult A.M.L. |
| Liposomal Cytarabine (DepoCyt)** | Lymphomatous meningitis |
| Liposomal Mifamurtide (Mepact)** | Osteogenic Sarcoma |

* A PEG-PLA polymeric micelle of paclitaxel, known as Genexol-PM is approved by the EMA as a generic version of Abraxane.

**DepoCyt and Mepact particle size is in the micron range, above the conventional size window of nanomedicines.

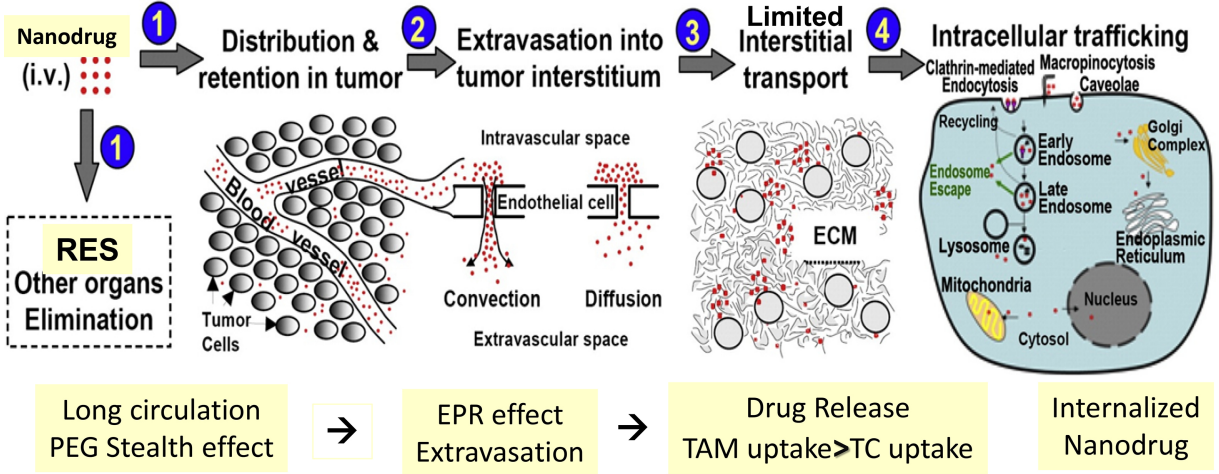

Nanodrug
(i.v.)
1
Distribution & retention in tumor
2
Extravasation into tumor interstitium
3
Limited Interstitial transport
4
Intracellular trafficking
1
RES
Other organs
Elimination
Blood vessel
vessel
Tumor Cells
Intravascular space
Endothelial cell
Convection
Diffusion
Extravascular space
ECM
Clathrin-mediated Endocytosis
Macropinocytosis
Caveolae
Recycling
Early Endosome
Golgi Complex
Endosome Escape
Late Endosome
Lysosome
Endoplasmic Reticulum
Mitochondria
Nucleus
Cytosol
Long circulation
PEG Stealth effect
EPR effect
Extravasation
Drug Release
TAM uptake>TC uptake
Internalized Nanodrug

Figure 1

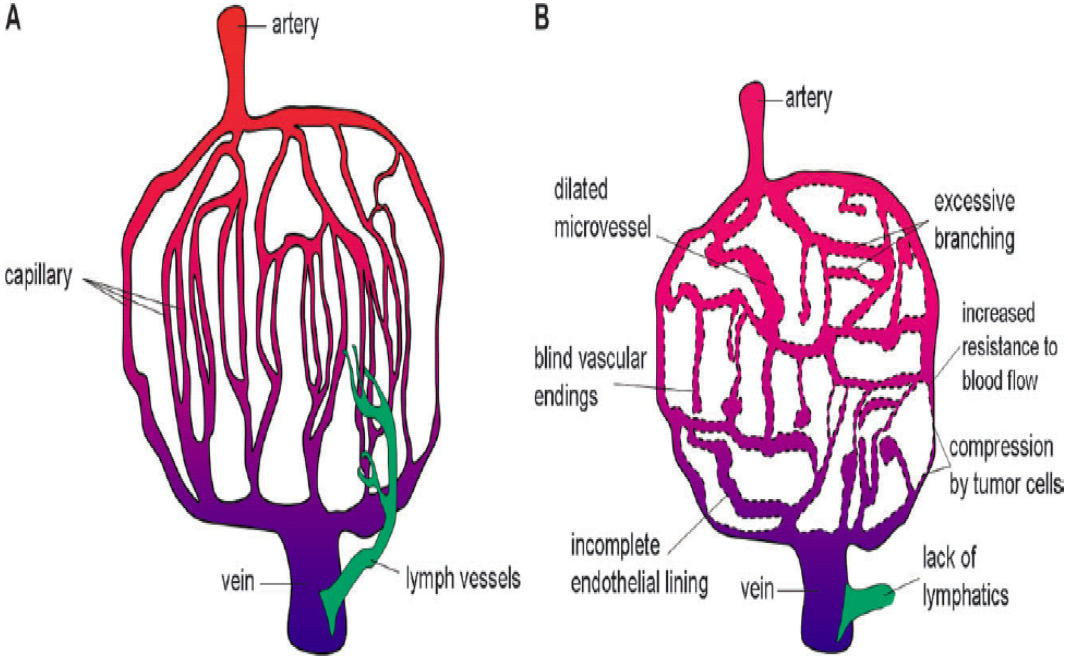

A
artery
capillary
vein
lymph vessels
B
artery
dilated
microvessel
excessive
branching
increased
resistance to
blood flow
blind vascular
endings
compression
by tumor cells
incomplete
endothelial lining
vein
lack of
lymphatics

Figure 2

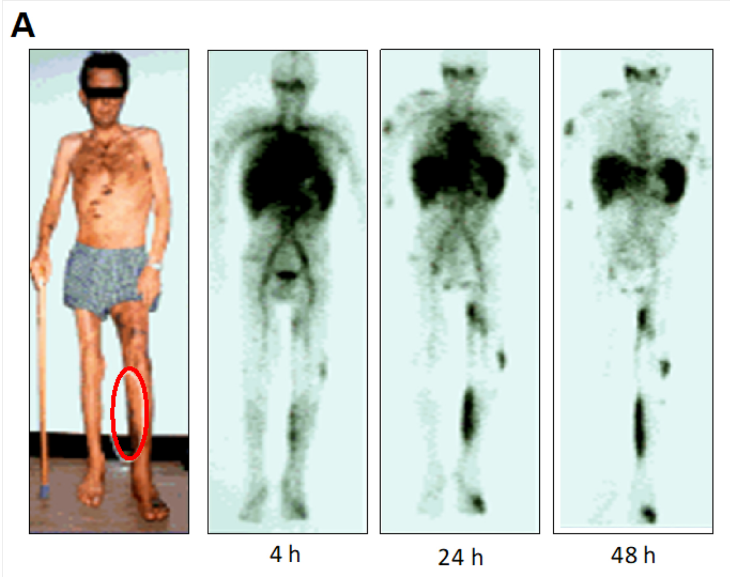

A
4 h
24 h
48 h
B
μg doxorubicin/gram
Dose:
10 mg/m2
20 mg/m2
40 mg/m2
Free doxorubicin
PLD
C
μg doxorubicin/gram
Dose:
10 mg/m2
20 mg/m2
Normal skin
Kaposi sarcoma

Figure 3

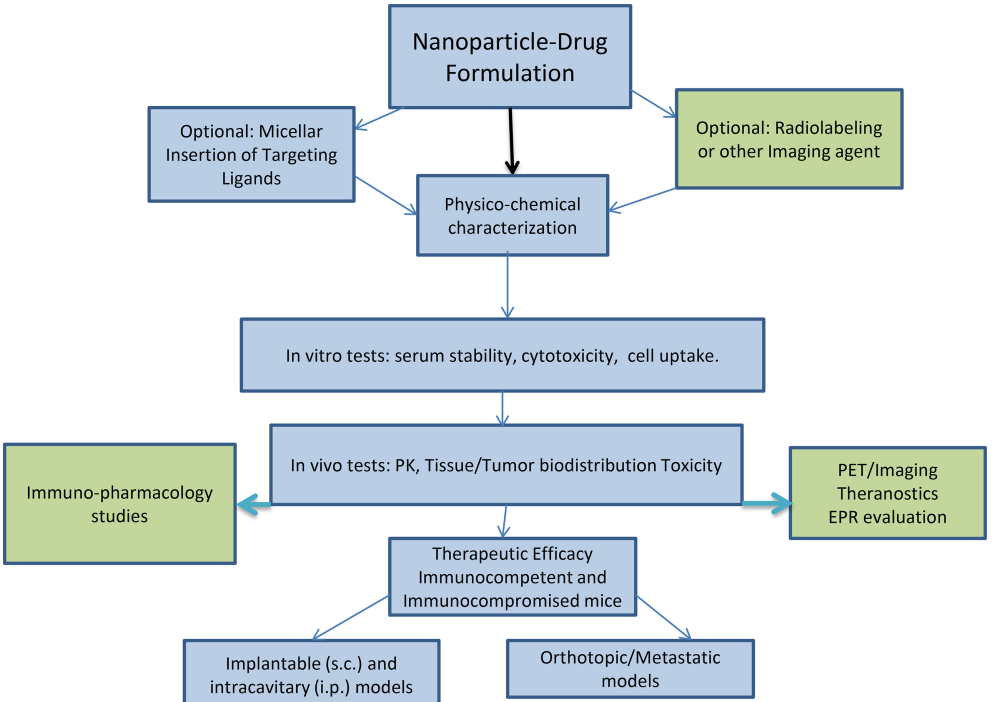

Nanoparticle-Drug Formulation
Optional: Micellar Insertion of Targeting Ligands
Optional: Radiolabeling or other Imaging agent
Physico-chemical characterization
In vitro tests: serum stability, cytotoxicity, cell uptake.
In vivo tests: PK, Tissue/Tumor biodistribution Toxicity
Immuno-pharmacology studies
PET/Imaging Theranostics EPR evaluation
Therapeutic Efficacy Immunocompetent and Immunocompromised mice
Implantable (s.c.) and intracavitary (i.p.) models
Orthotopic/Metastatic models

Figure 4

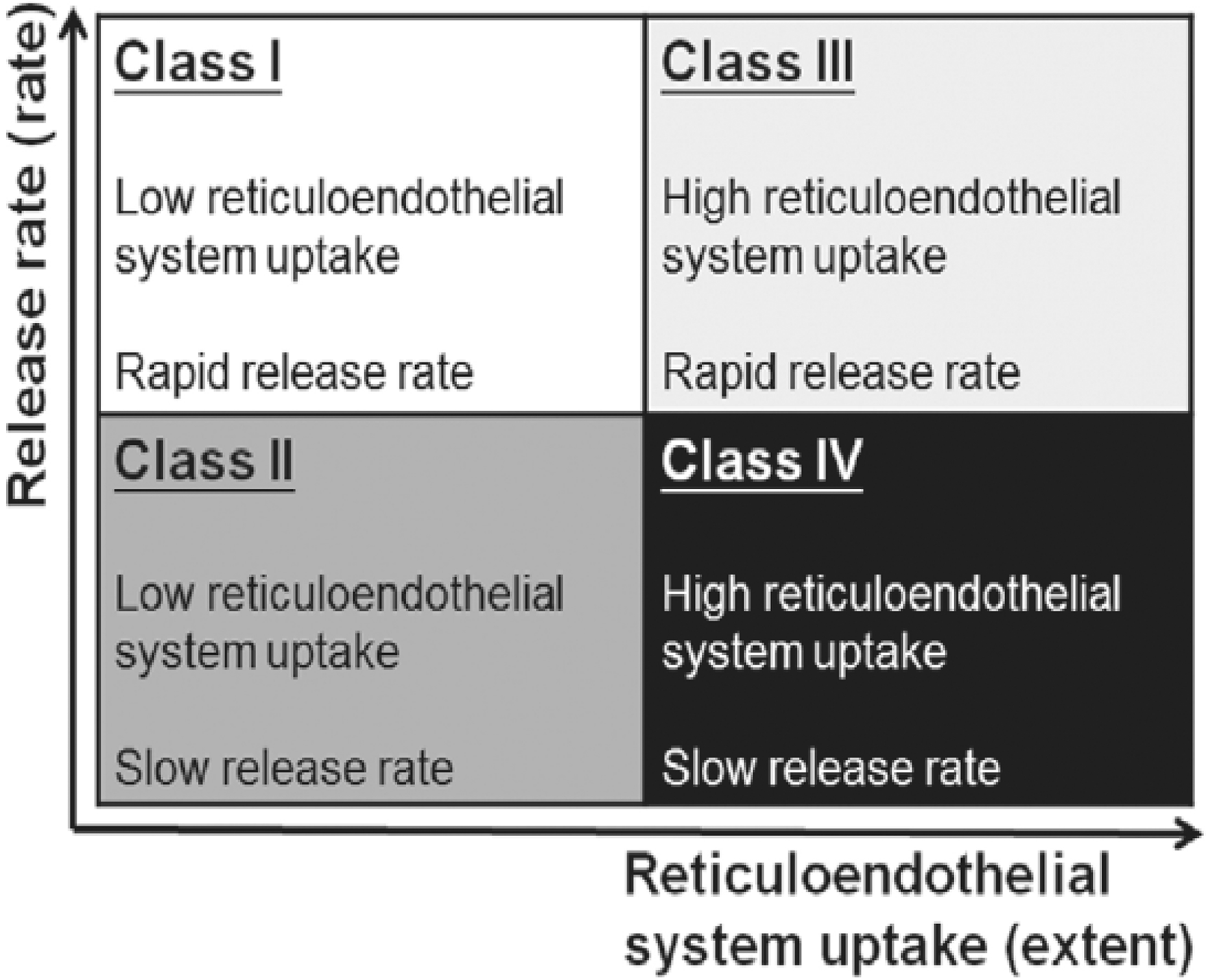
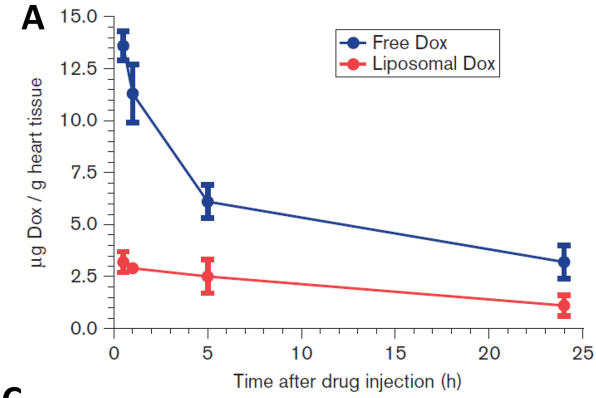
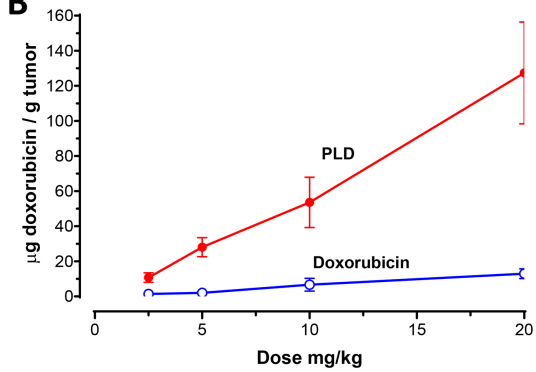
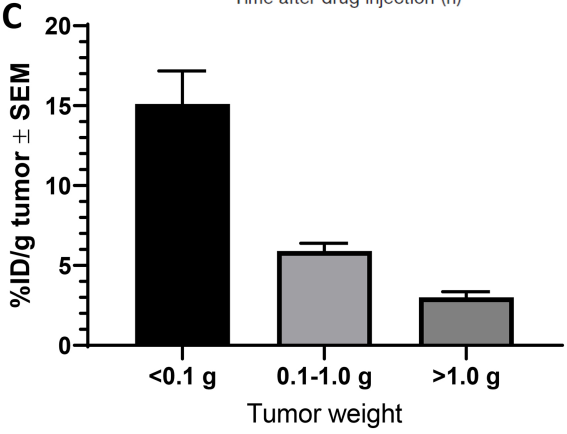

Release rate (rate)
Class I
Low reticuloendothelial system uptake
Rapid release rate
Class III
High reticuloendothelial system uptake
Rapid release rate
Class II
Low reticuloendothelial system uptake
Slow release rate
Class IV
High reticuloendothelial system uptake
Slow release rate
Reticuloendothelial system uptake (extent)

Figure 5


A
Free Dox
Liposomal Dox
µg Dox / g heart tissue
Time after drug injection (h)


B
µg doxorubicin / g tumor
PLD
Doxorubicin
Dose mg/kg


C
%ID/g tumor ± SEM
<0.1 g
0.1-1.0 g
>1.0 g
Tumor weight

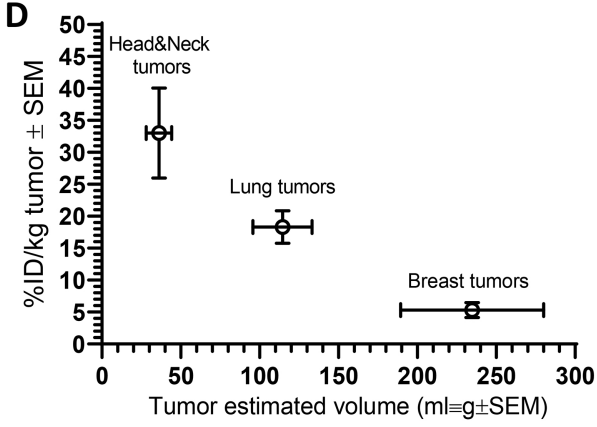
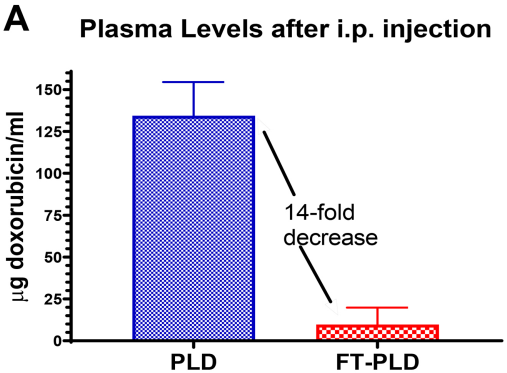
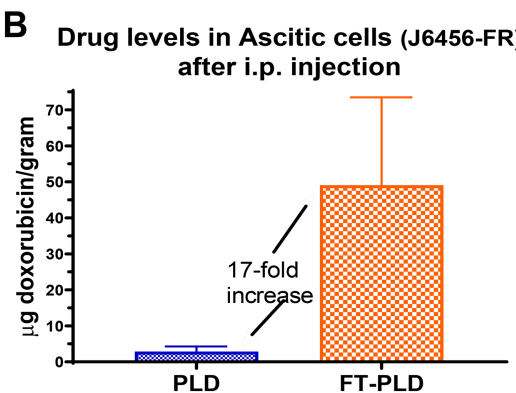

D
%ID/kg tumor ± SEM
Head&Neck tumors
Lung tumors
Breast tumors
Tumor estimated volume (ml≡g±SEM)

Figure 6


A
Plasma Levels after i.p. injection
µg doxorubicin/ml
150
125
100
75
50
25
0
14-fold
decrease
PLD
FT-PLD


B
Drug levels in Ascitic cells (J6456-FR)
after i.p. injection
µg doxorubicin/gram
70
60
50
40
30
20
10
0
17-fold
increase
PLD
FT-PLD

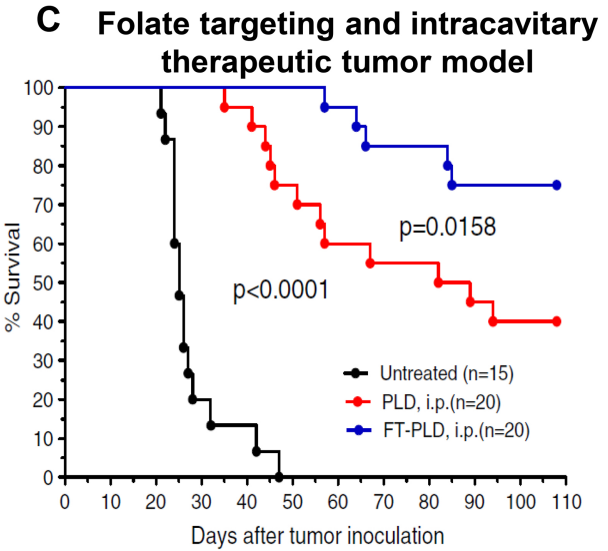

C
Folate targeting and intracavitary
therapeutic tumor model
% Survival
100
90
80
70
60
50
40
30
20
10
0
p=0.0158
p<0.0001
Untreated (n=15)
PLD, i.p.(n=20)
FT-PLD, i.p.(n=20)
0
10
20
30
40
50
60
70
80
90
100
110
Days after tumor inoculation

Figure 7

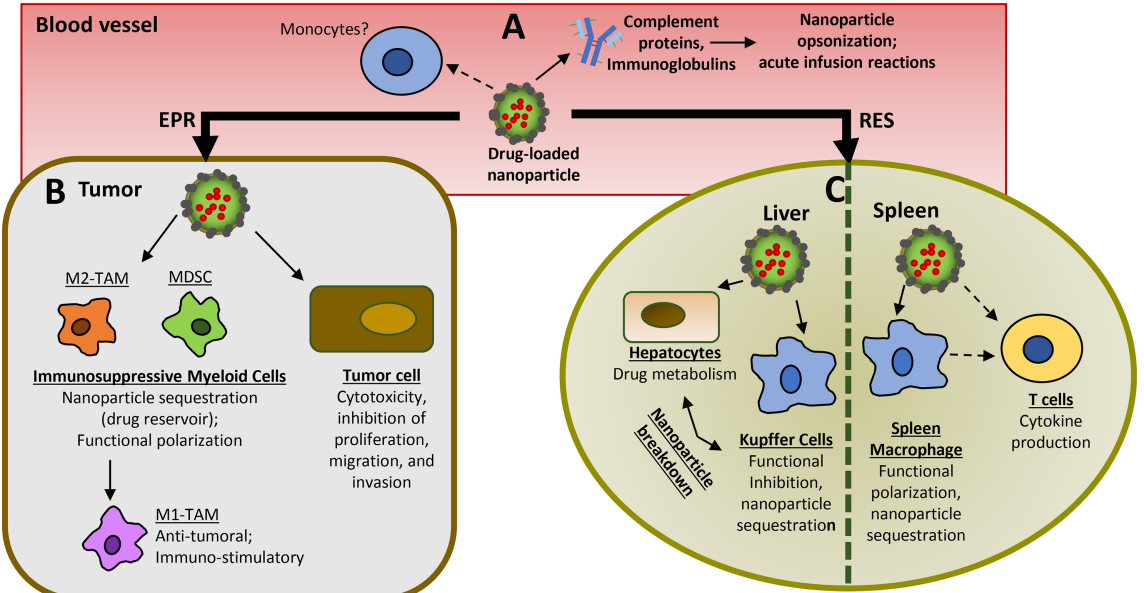

Blood vessel
Monocytes?
A
Complement proteins, Immunoglobulins
Nanoparticle opsonization; acute infusion reactions
EPR
RES
Drug-loaded nanoparticle
B Tumor
M2-TAM
MDSC
Immunosuppressive Myeloid Cells
Nanoparticle sequestration (drug reservoir); Functional polarization
Tumor cell
Cytotoxicity, inhibition of proliferation, migration, and invasion
M1-TAM
Anti-tumoral; Immuno-stimulatory
C
Liver
Spleen
Hepatocytes
Drug metabolism
Nanoparticle breakdown
Kupffer Cells
Functional Inhibition, nanoparticle sequestration
Spleen Macrophage
Functional polarization, nanoparticle sequestration
T cells
Cytokine production

Figure 8

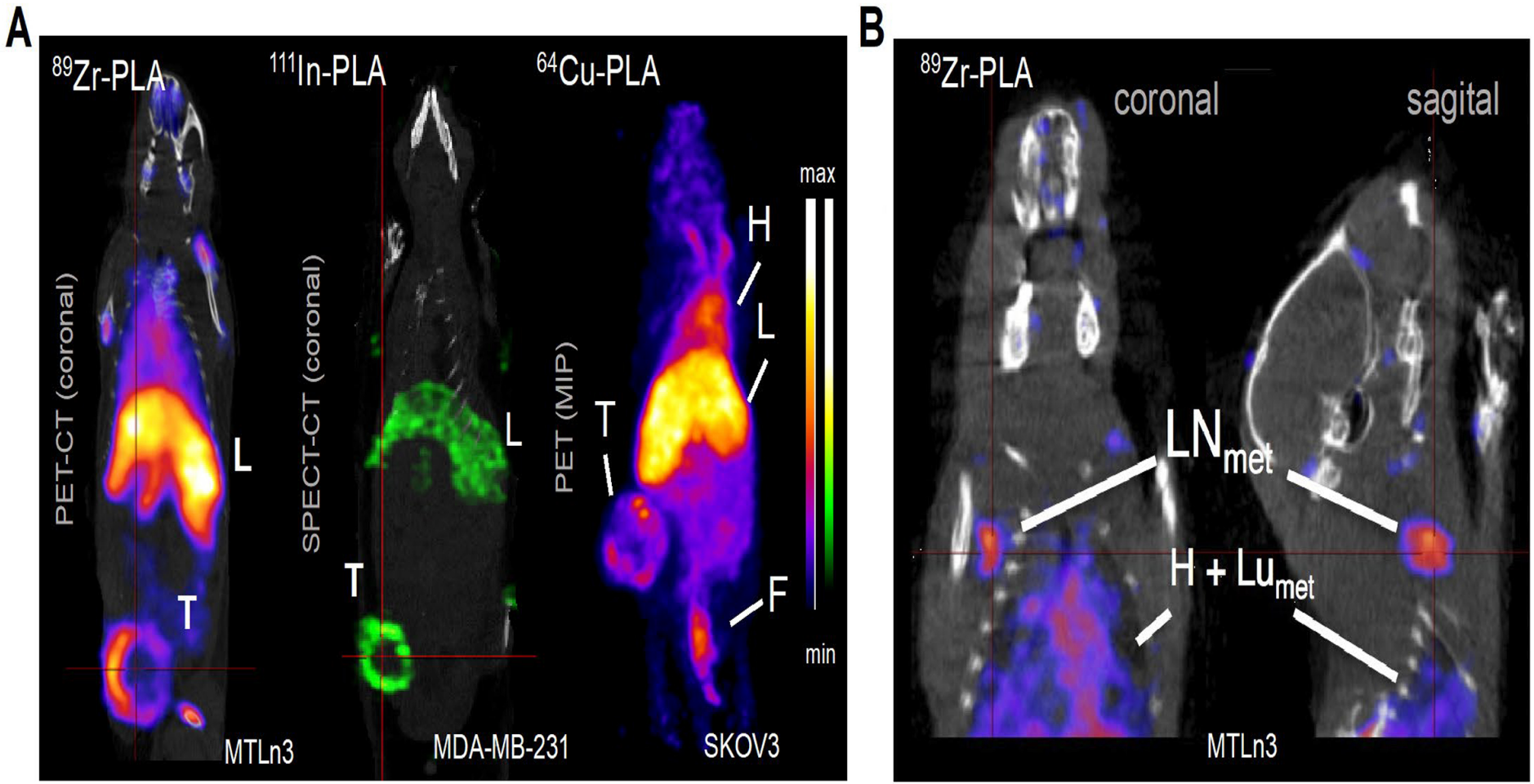

A
89Zr-PLA
111In-PLA
64Cu-PLA
PET-CT (coronal)
SPECT-CT (coronal)
PET (MIP)
L
T
L
T
H
L
T
F
max
min
MTLn3
MDA-MB-231
SKOV3
B
89Zr-PLA
coronal
sagital
LNmet
H + Lumet
MTLn3

Figure 9

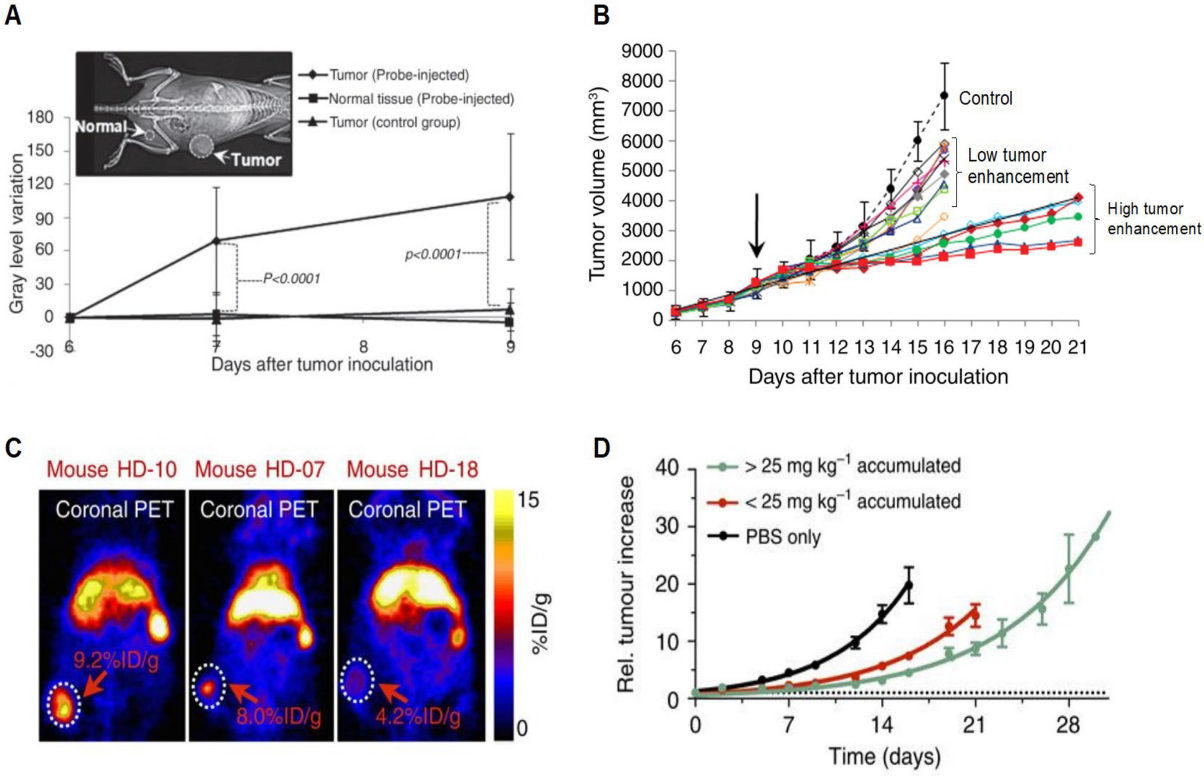

A
Tumor (Probe-injected)
Normal tissue (Probe-injected)
Tumor (control group)
Normal
Tumor
Gray level variation
180
150
120
90
60
30
0
-30
P<0.0001
p<0.0001
6
7
8
9
Days after tumor inoculation
B
Tumor volume (mm3)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Control
Low tumor enhancement
High tumor enhancement
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Days after tumor inoculation
C
Mouse HD-10
Mouse HD-07
Mouse HD-18
Coronal PET
Coronal PET
Coronal PET
9.2%ID/g
8.0%ID/g
4.2%ID/g
15
%ID/g
0
D
> 25 mg kg−1 accumulated
< 25 mg kg−1 accumulated
PBS only
Rel. tumour increase
40
30
20
10
0
0
7
14
21
28
Time (days)

Figure 10

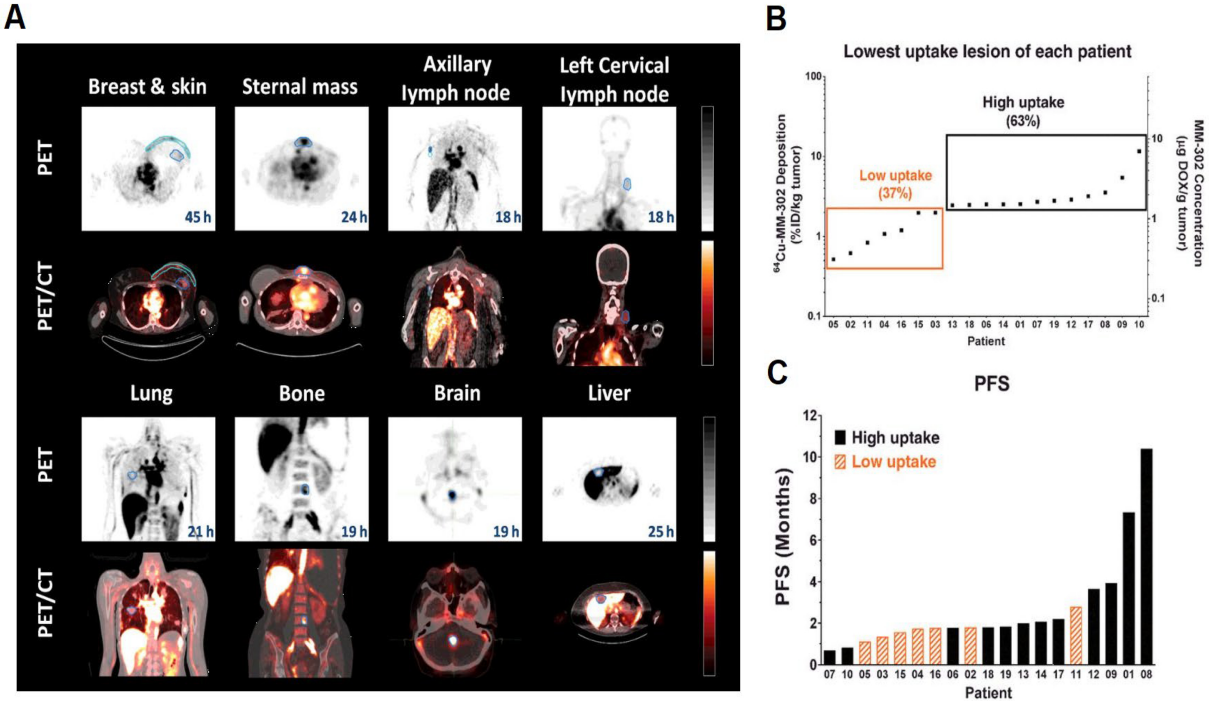
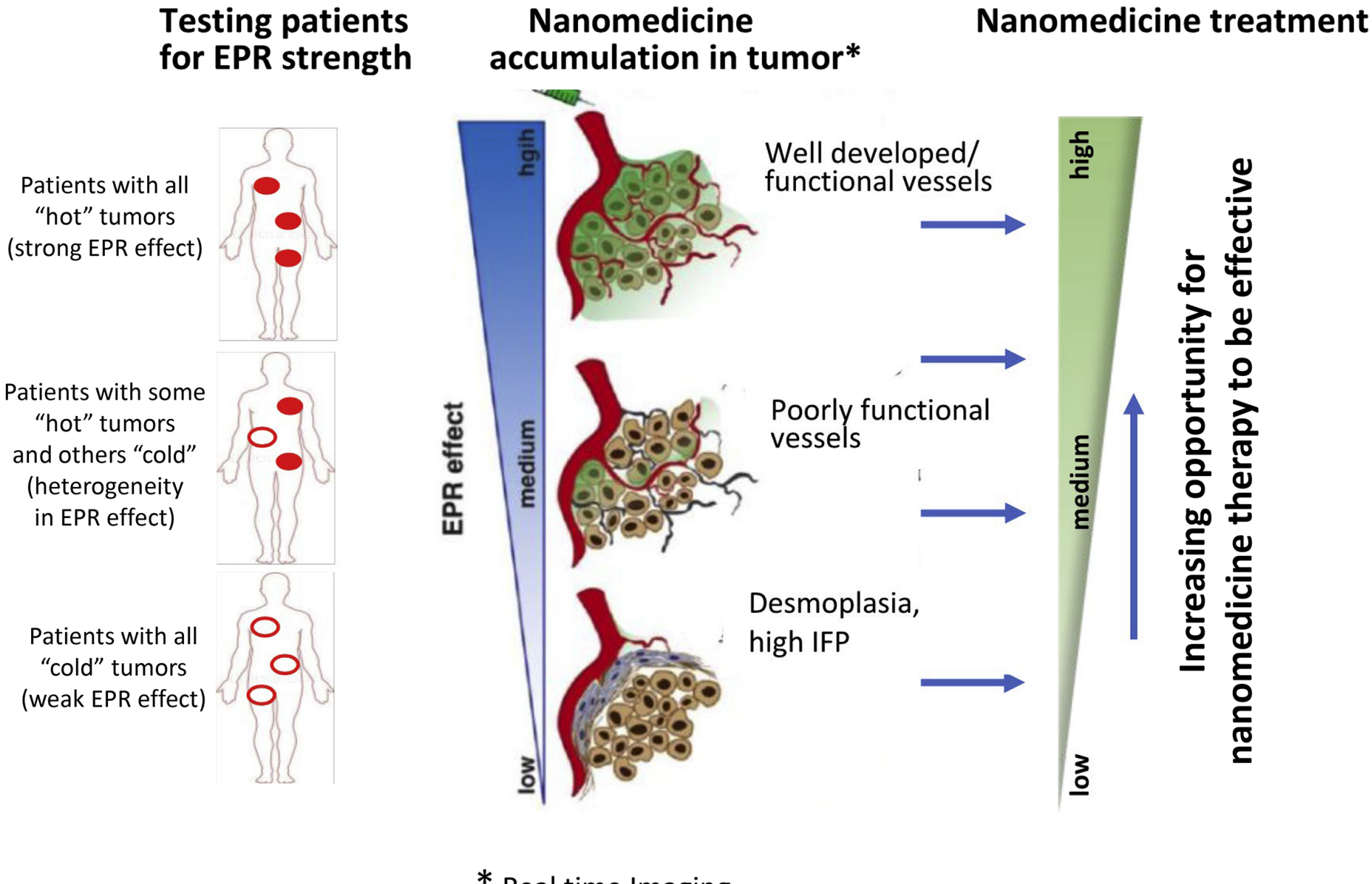

A
Breast & skin
Sternal mass
Axillary lymph node
Left Cervical lymph node
PET
PET/CT
45 h
24 h
18 h
18 h
Lung
Bone
Brain
Liver
21 h
19 h
19 h
25 h
B
Lowest uptake lesion of each patient
High uptake (63%)
Low uptake (37%)
64Cu-MM-302 Deposition (%ID/kg tumor)
MM-302 Concentration (μg DOX/g tumor)
Patient
05 02 11 04 16 15 03 13 18 05 14 01 07 19 12 17 08 09 10
C
PFS
High uptake
Low uptake
PFS (Months)
Patient
07 10 05 03 15 04 16 06 02 18 19 13 14 17 11 12 09 01 08

Figure 11




* Real time Imaging

Figure 12

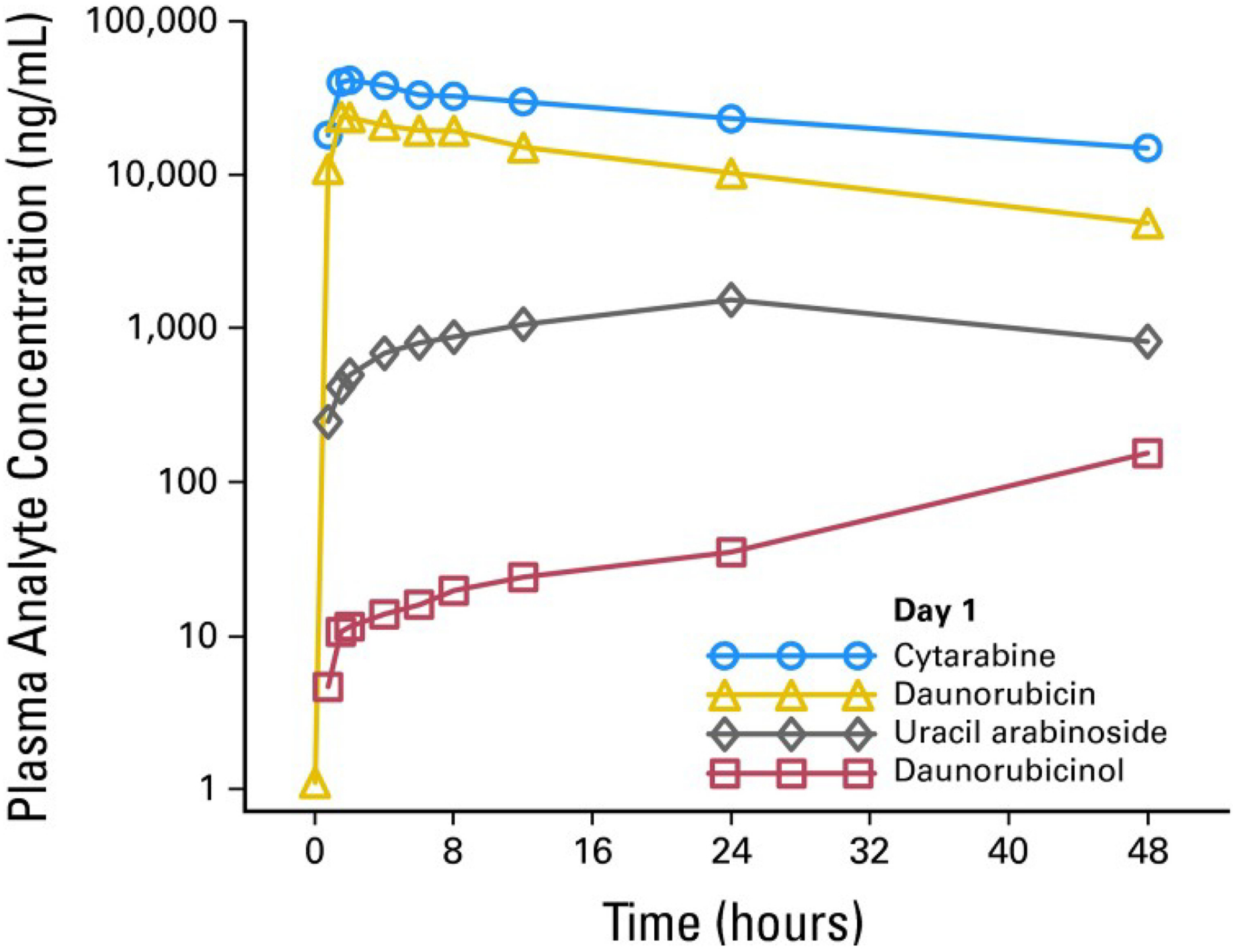

Plasma Analyte Concentration (ng/mL)
100,000
10,000
1,000
100
10
1
0
8
16
24
32
40
48
Time (hours)
Day 1
Cytarabine
Daunorubicin
Uracil arabinoside
Daunorubicinol

Figure 13