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Thirty years from FDA approval of pegylated liposomal doxorubicin (Doxil/Caelyx): an updated analysis and future perspective

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ABSTRACT

In 2025, it will be 30 years since the initial clinical approval of pegylated liposomal doxorubicin (PLD) by the Food and Drug Administration. PLD predated the field of nanomedicine and became a model nanomedicine setting key pharmacological principles (prolonged circulation, slow drug release and the enhanced permeability and retention (EPR) effect) for clinical application of other nano-drugs in cancer therapy. The impressive reduction of cardiotoxicity conferred by PLD is the most valuable clinical asset. While PLD has gained a strong foothold in relapsed ovarian cancer and metastatic breast cancer, it has not been extensively tested in primary (neoadjuvant) and adjuvant therapy and has not fulfilled the expectations from the results in animal models efficacy-wise. This discrepancy may be due to the large dose gap between mice and humans and the apparent variability of the EPR effect in human cancer. PLD is a complex product and we are still in a learning curve regarding a number of factors such as its interaction with the complement system and its immune modulatory properties, as well as its integration in multimodality therapy that may potentiate its value and role in cancer therapy.

EARLY WORK WITH LIPOSOMES AS DRUG DELIVERY SYSTEMS

Liposomes are submicroscopic vesicles consisting of an inner water phase surrounded by a bilayer of phospholipids usually mixed along with cholesterol, much alike the basic structure of cell membranes. The amphipathic character of phospholipids results in an orderly alignment of their phosphate moieties towards the internal and external water media, while their fatty acid chains create a lipophilic compartment within the bilayer (figure 1A). These lipid spherules can form spontaneously and were first described and characterised by Bangham and Horne[1] and Sessa and Weissmann[2] and served as models of membranes for biophysical studies.

Soon after the discovery of liposomes, Gregoriadis and Ryman proposed their use as carriers of drugs and enzymes particularly for lysosomal storage diseases.[3] Liposomes were considered to be ideal nanosized carriers for clinical use, given their biocompatibility, biodegradability, versatility and apparent lack of toxicity.[4 5] In a model of visceral leishmaniasis, Alving *et al* demonstrated a striking improvement of efficacy with reduced doses of antimonials encapsulated in liposomes.[6] This was an elegant proof of drug targeting to macrophages of the reticulo-endothelial system (RES) for which both liposomes and leishmania parasites have great affinity.

Besides their appeal as drug carriers, liposomes were also found to be safe and effective immunological adjuvants suitable for use in human vaccines.[7] Later on, it was reported that liposome encapsulation of antigens along with the inclusion of adjuvants such as lipid A or lipophilic muramyl dipeptide resulted in robust and safe vaccines.[8] Further studies by Fidler *et al* demonstrated that delivering macrophage-activating factors in large multilamellar vesicles (MLV) resulted in a significant activation boost of alveolar macrophages.[9]

During the initial rush to develop liposomes for medical applications, it was noticed that several physico-chemical factors contributed to wide variations in the liposome drug carrier pharmacological behaviour. These factors, listed in online supplemental table S1, are parameters established in the Food and Drug Administration (FDA) guidance to liposomal drug products and include the type of phospholipid (head, length of the fatty acid aliphatic chain and number of unsaturated bonds), the molar ratio of cholesterol to phospholipid (from 0:1 to 1:1), the shape and lamellarity (mutilamellar or unilamellar) as determined by cryogenic transmission electron microscopy (cryo-TEM), the liposome

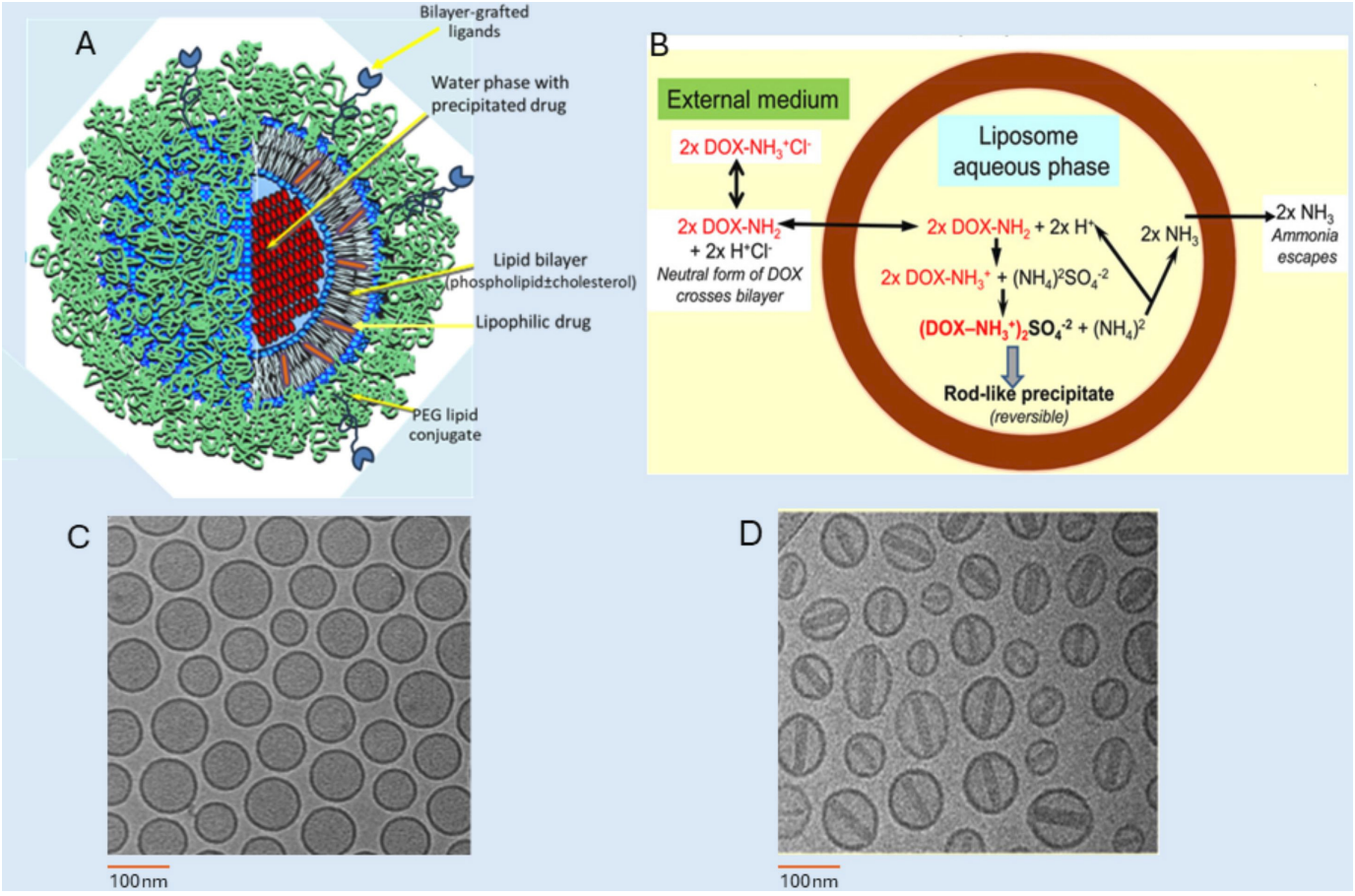

Figure 1 (A) Schematic diagram of a unilamellar liposome: liposomes are vesicular nanoparticles consisting of a phospholipid bilayer shell enclosing an internal water phase. Liposomes can serve as drug carriers whereby drug molecules are encapsulated in the water phase or entrapped in the lipid bilayer. Liposomes display high versatility in terms of size, composition, surface charge and lamellar structure. Liposomes may be coated with hydrophilic polymers such as polyethylene glycol (PEG) and decorated with ligands to specific receptors. (B) Mechanism of loading of doxorubicin (DOX) HCl in liposomes by the ammonium sulfate gradient for formulation of pegylated liposomal doxorubicin (PLD): liposomes are prepared in a highly concentrated ammonium sulfate buffer, followed by dialysis to remove external ammonium sulfate. DOX HCl is then added to the liposome suspension. As a cationic amphipathic drug, DOX can shuttle across the lipid bilayer in uncharged form; however, once inside the ammonium sulfate-rich water phase, it becomes protonated and is not able to cross back the bilayer, forming a salt with sulfate that leads to a reversible rod-like crystalline precipitate as the solubility limit of intraliposomal DOX sulfate is exceeded. In parallel, the ammonium ion (NH4+) dissociates to bilayer-permeable and volatile NH3 and provides protons that are critical to maintain intraliposomal DOX in protonated form. Preferably, the loading process takes place at a high temperature above the Tc of the lipid bilayer. (C) Cryogenic transmission electron microscopy (TEM) of liposomes before drug loading: liposomes are spherical and uniform with a diameter of 80–100 nm. (D) Cryogenic TEM of drug-loaded PLD liposomes: characteristic coffee bean appearance due to precipitation of DOX sulfate and formation of rods that result in a distorted ovoid shape.

liquid-crystalline phase transition temperature (Tc) as measured by differential scanning calorimetry, the liposome surface charge as measured by the zeta potential and the liposome size (Z-average diameter) and polydispersity index as determined by dynamic laser scattering. The smallest liposomes that can be obtained, regardless of the process of manufacture, are ~30 nm diameter, although in most instances the liposomes used for intravenous administration in the clinic range in size between 70 and 200 nm.

Because of the presence of an aqueous and a lipid phase in the liposome, it is possible to entrap both hydrophilic and lipophilic substances (figure 1A), thus providing the opportunity to deliver a broad array of active pharmaceutical ingredients (API) with widely diverse chemical composition and physical properties.[10]

LIPOSOMAL DOXORUBICIN: THE HYPOTHESIS FOR REDUCED CARDIOTOXICITY AND LESSONS FROM EARLY HUMAN STUDIES

In the early 1980s, several investigators reported that the use of liposomes to deliver doxorubicin could reduce doxorubicin uptake by the heart muscle and along with it the infamous cardiac toxicity of this potent chemotherapy agent widely used for treatment of many types of cancer.[11–16] Our hypothesis for this cardiac toxicity-sparing

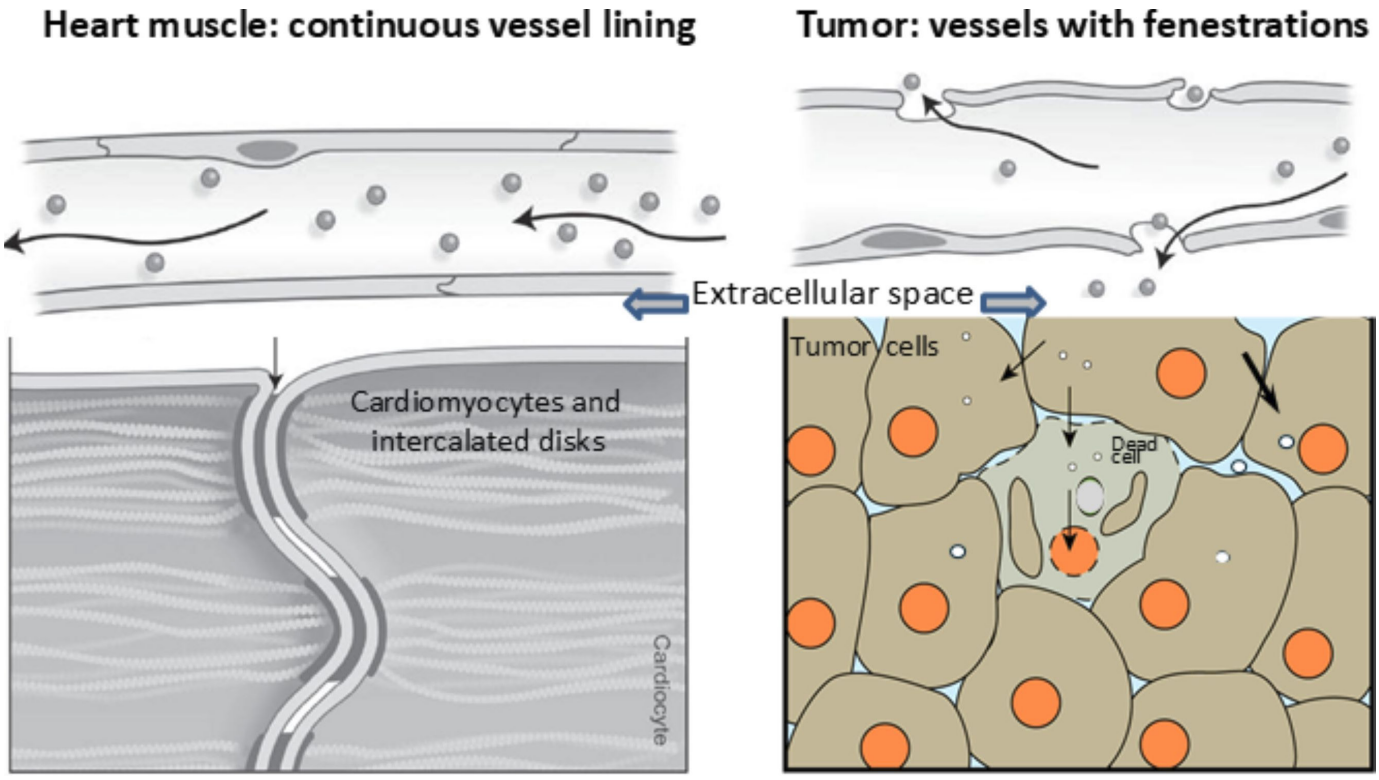

Figure 2 Pharmacological rationale for pegylated liposomal doxorubicin—heart versus tumour: liposomes (small circles) cannot gain access to interstitial space of the myocardium due to tight junctions of microvessel endothelium (left panel), while they can traverse the fenestrations of pathological tumour microvessels and enter the tumour compartment (right panel).

effect was simply that the micro-anatomy of the heart muscle vessels does not allow for penetration of particles such as liposomes (ie, around 100 nm), while that of tumour vessels is sufficiently porous to allow extravasation of liposomes (figure 2). Therefore, as long as doxorubicin is retained by circulating liposomes, the drug will be unable to reach the cardiomyocytes and exert its toxic effect. Obviously, liposomes still have to reach the tumour tissue in the primary and metastatic sites to deliver doxorubicin at effective pharmacological levels and achieve a significant antitumour effect. One confounding factor was the fact that liposomal doxorubicin is generally less toxic than free doxorubicin in mice, thereby improving the therapeutic index and the overall efficacy. Clearly, if the dose has to be raised to obtain superior efficacy, this was far from an ideal approach because the toxicity-sparing effect would be lost. Further data analysing the fate of systemically administered liposomes suggested that, apart from liver and spleen, organs with discontinuous (sinusoidal) capillaries, liposomes are unable to escape from continuous capillaries calling into question the feasibility of using liposomes to 'target' drugs to tumour cells in extravascular tissues.[17] Yet, tumours diffusely spread to the liver could be efficiently targeted with conventional liposomes, as shown with a mouse lymphoma model in which liposomes could deliver doxorubicin to tumour cells at far greater levels than free doxorubicin, resulting in superior therapeutic activity.[18 19]

Most of the initial work with doxorubicin liposomes focused on formulations containing negatively charged phospholipids (cardiolipin, phosphatidylserine, phosphatidylglycerol (PG)) to enhance the entrapment efficiency of doxorubicin in the lipid bilayer by electrostatic interaction with its positively charged amino sugar moiety, daunosamine. While these negatively charged formulations caused a significant change in pharmacokinetics (PK) and biodistribution (BD) of doxorubicin in mice, they were insufficient to make a substantial pharmacological impact in humans. We conducted a phase I study with a negatively charged (PG-containing) liposome formulation of doxorubicin in 32 patients with advanced cancer.[20] While the maximal tolerated dose (MTD) of liposomal doxorubicin (~100 mg/m^2) exceeded the MTD of free doxorubicin (75 mg/m^2) in the standard three-weekly schedule, disappointingly the subacute toxicity was similar to that of free doxorubicin (myelosuppression, stomatitis, alopecia). The PK and imaging part of this first-in-man study provided valuable information on the need for improvement in two areas[21]:

1. *Improving the retention of doxorubicin in liposomes while in circulation*: plasma clearance of total drug in patients receiving liposomal doxorubicin followed a biexponential curve with a pattern similar to that reported for free drug. In addition, liposome

clearance, as measured by the plasma concentration of PG, a phospholipid liposome component, was relatively slower than the clearance of liposome-associated drug, indicating that liposomes lose part of their drug payload during circulation. The failure of negatively charged PG-containing liposomes with doxorubicin intercalated in the lipid bilayer to demonstrate stability in human PK studies brought up the need to remedy this with an efficient water phase loading method to be discussed in the section "Stealth liposomes: the pharmacological value in cancer of long-circulating, stable, liposomal drug carriers".

2. *Reducing the fast hepato-splenic clearance of liposomes*: ^{111}Indium-deferoxamine-labelled PG-containing liposomes were cleared predominantly by liver and spleen and to a lesser extent by bone marrow. Except for one patient with hepatoma, intrahepatic and extrahepatic tumours were not imaged by liposomes, suggesting rapid liposome clearance and uptake restricted to the RES.

These observations on the lack of in vivo stability with our early formulation of liposomal doxorubicin were echoed by another parallel phase I study in 14 patients with advanced cancer with a hydrophobic cytostatic agent (NSC 251635), entrapped in positively charged stearyl-amine-containing liposomes,[22] which also found a rapid dissociation of the drug from liposomes in circulation. The authors were also the first to report an important activation of the complement system in all patients investigated, possibly associated with the presence of positively charged stearyl-amine.[23]

STEALTH LIPOSOMES: THE PHARMACOLOGICAL VALUE IN CANCER OF LONG-CIRCULATING, STABLE, LIPOSOMAL DRUG CARRIERS

Until the end of the 1980s, except for the significant reduction of cardiac toxicity of anthracyclines, liposomes did not offer any appeal for drug delivery breakthroughs in cancer. Several studies had identified factors that modify favourably the circulation time, stability and permeability of liposomes: small vesicle size,[24 25] inclusion of cholesterol, and a higher phospholipid Tc or replacement with sphingomyelin.[26 27]

In 1986, Matsumura and Maeda reported on the tumour accumulation of macromolecules due to enhanced blood vessel permeability and poor drainage by lymphatic vessels.[28] This passive targeting effect of macromolecules to tumours was later known as the enhanced permeability and retention (EPR) effect and observed with liposomes and other nanoparticles. An analysis of the clearance of 20 different liposome compositions, all with a mean size of 100 nm, in normal and tumour-bearing mice demonstrated a correlation between long circulation time, low accumulation in RES (liver and spleen) and a major increase of liposome concentration in the tumour.[29] These data supported the efforts to formulate liposomes with long circulation times for a valuable targeting and therapeutic effect of liposomal drugs. While adding a small fraction of sugar-containing lipids was a very effective means to increase circulation time,[29] pegylation, i.e. the surface coating of liposomes with a small fraction of a polyethylene glycol (PEG) polymer-lipid conjugate, became the first choice for pharmaceutical reasons and rapid clinical translatability. In addition, pegylation of proteins had become well established[30] and, therefore, was quickly applied to liposomes.[31–33]

In addition to circulation time, another critical factor to ensure the in vivo performance of this new generation of liposomes, referred to as Stealth liposomes,[34] was stable drug retention in circulation. In fact, long circulation requires a higher threshold of stability to ensure drug retention during a longer residence in circulation. In addition, the greater dilution effect when liposomal drugs are injected in humans, as opposed to small rodents, generates a stronger diffusion gradient that compromises drug retention.[35] Two significant breakthroughs in remote loading methodology (ie, loading drugs into preformed liposomes from an outside buffer) to encapsulate cationic amphipathic drugs, such as doxorubicin, solved the problem of in vivo stability. Mayer *et al* found that doxorubicin can be rapidly and efficiently accumulated into liposomes in response to a transmembrane pH gradient in which the liposome interior is acidic,[36] and later demonstrated that along with the use of distearoylphophatidylcholine (DSPC), a high Tc phospholipid, as main liposome component, in vivo stability was improved and toxicity reduced in mice.[37] Furthermore, Barenholz *et al* developed an ingenious method of remote loading based on an ammonium sulfate gradient (figure 1B), which does not require preparation of the liposomes in acidic pH or alkalinisation of the extra-liposomal aqueous phase and is the preferred method used in the pharmaceutical manufacture of Doxil/Caelyx and generics of pegylated liposomal doxorubicin (PLD).[38] This method results in the formation of intraliposomal rods of a doxorubicin-sulfate precipitate that elongates the liposome shape from spherical to oval shape resulting in the characteristic 'coffee bean' structures seen by cryo-TEM (figure 1C-D).[39] Stealth liposomal doxorubicin prepared with this method was soon proven to perform well in vivo in mice and dogs with regard to all parameters (PK, stability, toxicity and/or therapeutic efficacy),[40 41] with remarkably high tumour drug uptake in mouse tumour models (figure 3A-C) and in some patients with cancer (figure 3D).[42]

While Stealth liposomal doxorubicin is extremely stable in plasma, BD data indicate that liposomal drug is released gradually in the tumour bed.[42] The tumour interstitial fluid and the cellular composition of the tumour microenvironment (TME) including the presence of liposome-engulfing, tumour-associated macrophages (TAM), expose liposomes to a totally different milieu than plasma, which accelerates liposome breakdown and drug

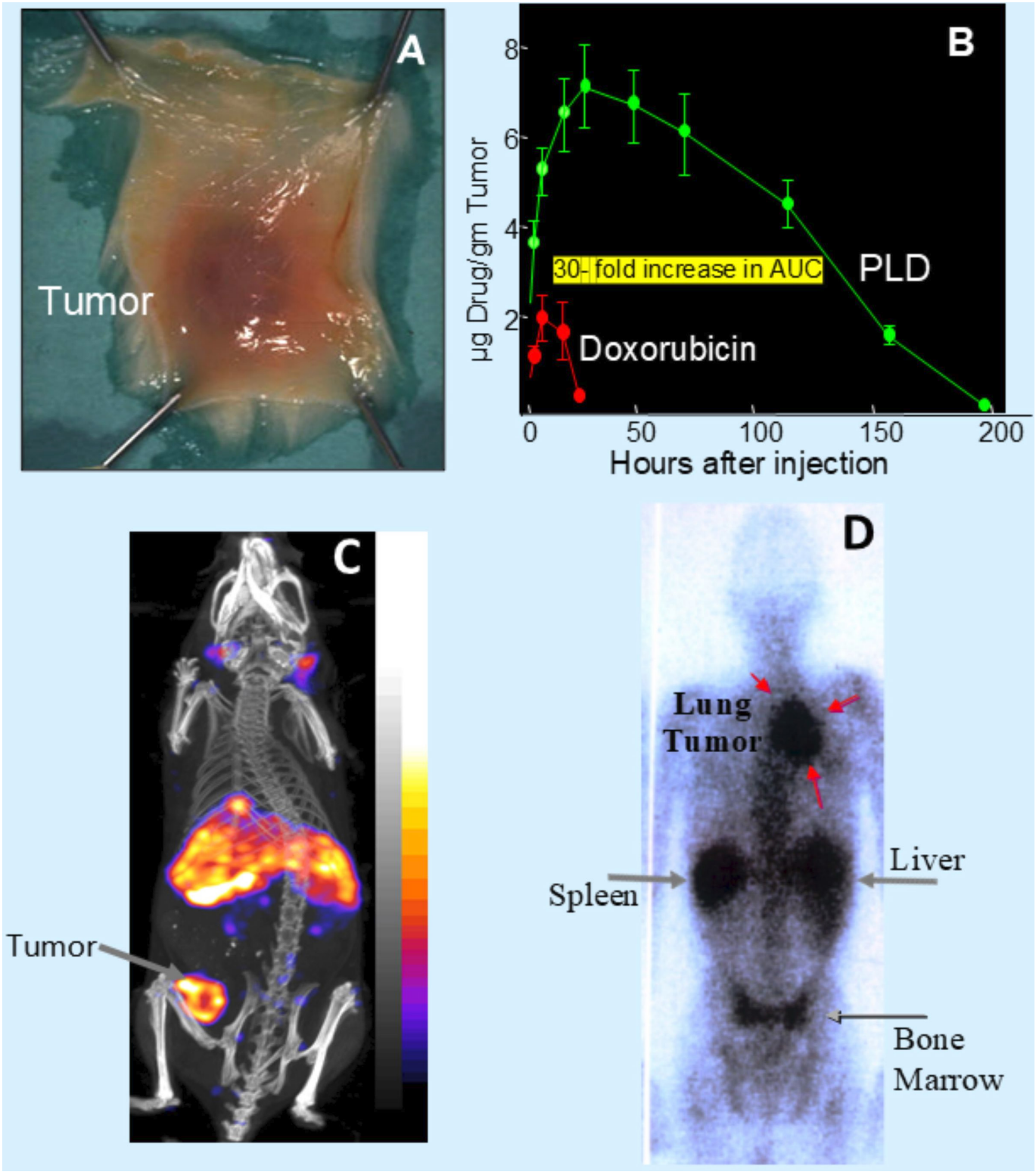

Figure 3 (A) Marked doxorubicin deposition in mouse tumour implants: 48 hours after an intravenous PLD dose of 20 mg/kg, the tumour drug concentration is 86 µg/g (~22% ID/g) and the red color of doxorubicin is recognizable by the naked eye, while in the normal skin (sample from the flank), drug concentration is 0.5 µg/g, that is, more than a 100-fold drop. (B) Time plot of accumulation of PLD and free doxorubicin in human tumour mouse model: note the 30-fold increase in tumour drug area under the curve (AUC) and the delayed peak tumour concentration when PLD is compared with free doxorubicin. (C) Passive targeting (EPR) of PLD-like liposomes in human breast cancer model in nude (immunodeficient) mice: mice bearing MDA-MB-231 breast cancer tumours imaged with ^{111}indium-labelled liposomes. Tumour uptake reached 25% of injected dose per gram, surpassing liver uptake, and second only to spleen uptake. (D) Accumulation of ^{111}indium-labelled pegylated liposomes in human lung tumour (posterior view): note that tumour uptake is comparable to liver and spleen. In addition, substantial uptake is also visualised in the bone marrow of pelvis. Adapted from Solomon and Gabizon[145]; Vaage *et al.*[146]; Man *et al.*[147]; Harrington *et al*.[45].

release. In addition, metabolic features of cancer cells, such as glutaminolysis, may affect the gradient holding doxorubicin in liposomes and accelerate its release, as proposed by Silverman and Barenholz.[43] Therefore, while the ammonium sulfate gradient loading method will surely slow down drug release in the tumour site, it is unlikely that it will result in a significant decrease of drug bio-availability in tumour tissue.

Another critical aspect for the success of Stealth liposomal drug delivery is the validation of the EPR effect in humans. While EPR is observed consistently in experimental tumour models (figure 3A–C), large variations have been observed in human cancer as reviewed by Man *et al*.[44] Back in 2001, Harrington *et al* observed a large interpatient variation between 2.7% and 53.0% ID/kg, based on scintigraphic studies with ^{111}In-labelled Stealth liposomes and volumetric estimates of tumours,[45] in agreement with a later study with ^{64}Cu-labelled HER2-targeted PLD.[46] Direct contributing factors to EPR variability include tumour type, tumour size and tumour site (primary vs metastatic tumours). Mechanistically, the underlying factors of EPR variability are related to the microanatomy of tumour blood vessels, the presence and number of TAM, the abundance and stiffness of the extracellular matrix present in the TME and the tumour interstitial fluid pressure (IFP). The presence and/or pharmacological impact of EPR in human cancer remains a controversial subject.[47] Efforts at enhancing the EPR effect with concomitant medications or physical methods are ongoing.

INITIAL CLINICAL-PHARMACOKINETIC STUDIES WITH PEGYLATED LIPOSOMAL DOXORUBICIN

In 1994, we published the results of a first-in-man study with PLD in patients with cancer comparing the PK of PLD and free doxorubicin sequentially in the same group of patients.[48] The PK of doxorubicin were drastically altered after administration of PLD with a 1000-fold increase of plasma area under the curve (AUC) and followed a profile dictated by the liposome carrier, as indicated by the superimposable clearance curves of total doxorubicin and liposome-associated doxorubicin after PLD administration (figure 4A,B). For detailed information on the PK parameters of PLD, see Gabizon *et al*.[49] As expected, a PK change of such unprecedented magnitude for a systemically administered drug had a significant pharmacodynamic (PD) impact reflected in a major change of the toxicity profile of PLD when compared with free doxorubicin (table 1). The unique toxicity profile of PLD was observed in two complementary phase I studies in the USA and Israel,[50] which revealed that skin toxicity manifested primarily as hand-foot syndrome also known as palmar-plantar erythrodysesthesia (PPE) and stomatitis are the two main dose-limiting factors of PLD, while subjective symptoms, myelosuppression, alopecia and particularly cardiotoxicity were much attenuated. PPE was dose-limiting for repetitive dosing, and more effectively prevented by increasing dose interval rather than reducing dose. The single-dose MTD was established at 60 mg/m^2 every 4 weeks and was lower than that of free doxorubicin, in contrast to the data from rodent studies where PLD was ~twofold less toxic than the free drug.[40] The reason for this discrepancy remains unclear. One possible explanation is that the faster clearance of PLD in mice as compared with humans (T½, 15–20 hours in mice and 55–75 hours in most patients) with faster sequestration of PLD in the RES may allow the former to buffer more effectively the mucocutaneous toxicities.

There is some degree of interpatient variability in the PK of PLD, mostly age and gender related. Children and adult male patients have faster clearance than adults and females, respectively.[51 52] Although there are no formal recommendations, clinicians should consider dose reduction when treating elderly women.

The heart muscle sparing of PLD became clear as more and more patients received large cumulative doses without any evidence of cardiotoxicity despite reaching cumulative doses close to 1500 mg/m^2, approximately threefold greater than the maximal cumulative dose of free doxorubicin allowed.[53] This was confirmed in a small group of patients undergoing electron microscopy examination of endomyocardial biopsies.[54] The cardiac safety of PLD is probably its most valuable hallmark for clinicians, enabling continuous treatment for long periods of time with a record-breaking case of 9 years and 4600 mg/m^2 cumulative dose.[55]

Along the first decade after clinical approval of PLD, several early phase clinical studies added important observations on the PK-PD relationship of PLD. The average Cmax, rather than the AUC or T½, was found to be the best predictor of response in patients with Kaposi's sarcoma (KS).[56] In a phase I/II breast cancer study, a correlation between Cmax and leucopenia/neutropenia and stomatitis, and between T½ and skin toxicity, mainly PPE, became evident.[57] Furthermore, PLD appears to cause a subtle damage to the RES clearance mechanism, which is manifested by an increase of AUC of 43% in subsequent cycles of PLD, suggesting that a rational treatment strategy is to start with a relatively high dose and de-escalate thereafter to avoid or attenuate mucocutaneous toxicities.[58] In a secondary analysis of phase I/II patients treated with PLD, it was found that age (elderly patients), gender (women) and low monocyte counts correlate with slower PLD clearance.[51] Interestingly, combining PLD with cisplatin, and possibly also other platinum agents, accelerates clearance of PLD, without affecting Cmax, and was well tolerated with low incidence of skin toxicity.[59] In contrast, an interference with PLD clearance was observed when taxanes are given alongside PLD.[60] These PK observations may explain the successful application of the carboplatin-PLD combination in gynaecological cancers and suggest that taxanes and PLD should be given staggered rather than concomitantly.

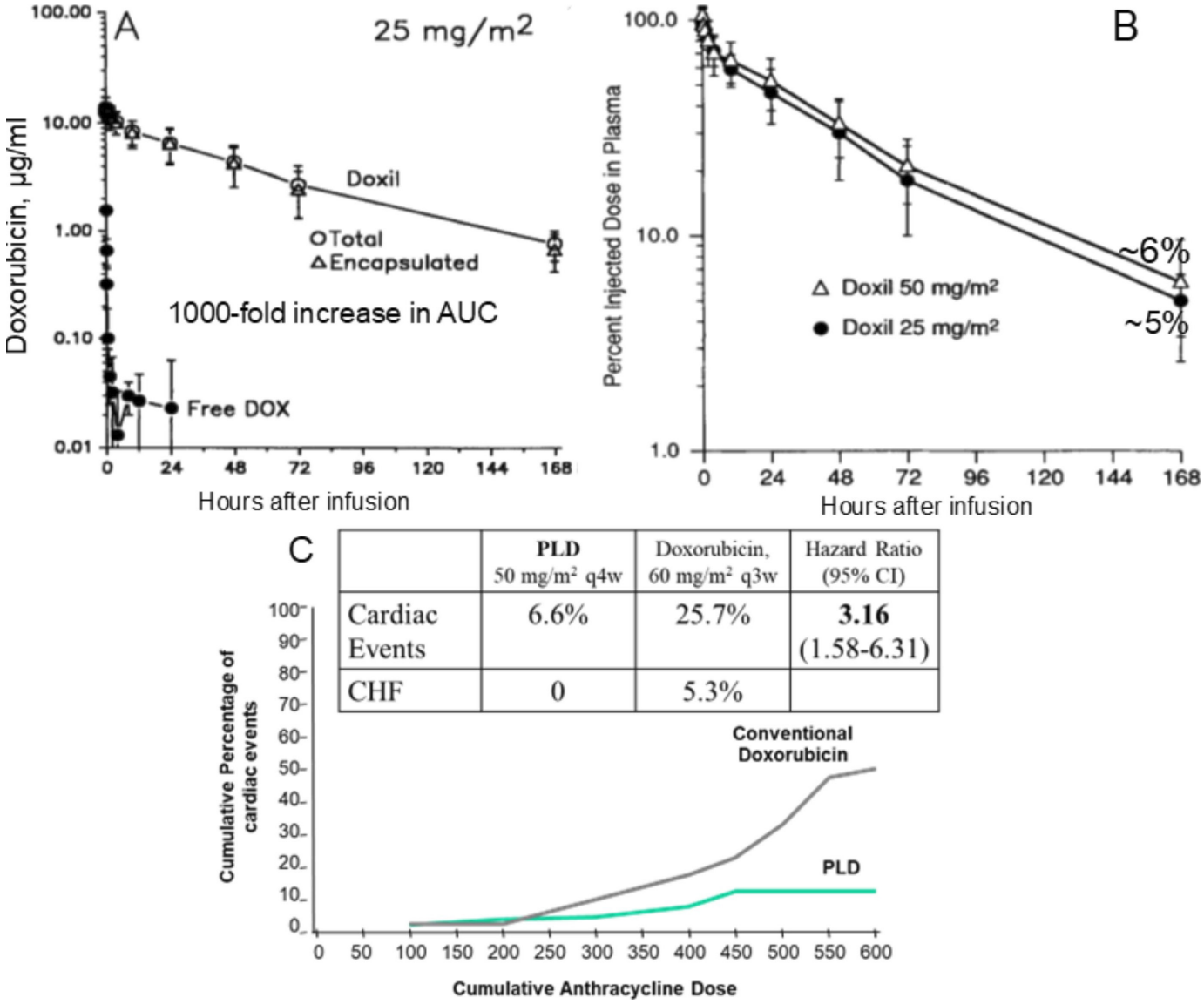

Figure 4 (A) Pharmacokinetics of pegylated liposomal doxorubicin (PLD) and free doxorubicin (DOX) in humans: both treatments were administered at a dose of 25 mg/m^2, 3 weeks apart, in a group of 15 patients with cancer. Note the huge differences in AUC and clearance. (B) Time curve of percent injected dose of PLD in plasma: note the dose-independent quasi mono-exponential pharmacokinetics within the dose range of 25–50 mg/m^2. On day 7 after infusion, 5%–6% of the injected dose is still present in plasma. (C) Cumulative percentage of cardiac events versus cumulative dose of PLD: the risk of developing a cardiac event was significantly lower with PLD than conventional DOX with an HR of 3.16 in favour of PLD (see inset table). At all cumulative doses >450 mg/m^2, the cardiac risk for patients treated with PLD did not increase. Patients in the PLD and DOX arms had a median cumulative anthracycline dose of 400–420 mg/m^2 including prior anthracycline exposure. In the subgroup that received prior adjuvant anthracycline therapy, the risk of developing cardiotoxicity was sevenfold higher with DOX than with PLD. None of the 10 PLD-treated patients who had cardiotoxicity by left ventricular ejection fraction (LVEF) criteria developed clinical signs or symptoms of congestive heart failure (CHF, whereas 10 of 48 DOX-treated patients who had cardiotoxicity by LVEF criteria developed signs or symptoms of CHF. Adapted from: Gabizon *et al*[48]; O'Brien *et al*.[74]

THE CLINICAL PATH OF PLD: APPROVALS FOR KAPOSI'S SARCOMA, RECURRENT OVARIAN CANCER, METASTATIC BREAST CANCER AND MULTIPLE MYELOMA

The added clinical value of PLD over standard of therapy was initially demonstrated in AIDS-related KS. The AIDS epidemics of the 1980s, when antiretroviral drugs were still lacking, resulted in a high incidence of aggressive KS. Given the high vascular permeability of KS skin lesions, it was very logical to focus the clinical development of PLD on this unmet need. Several randomised clincial trials demonstrated doubling of objective response rate (ORR) in patients receiving PLD compared with conventional therapy including doxorubicin, bleomycin and vincristine (ABV). Two major studies compared PLD with either BV or ABV.[61 62] Both demonstrated a significant difference in overall response, shorter time to response and clinical improvement in patients with KS. Thus, overall PLD was found to be the most effective agent for AIDS-related KS

Table 1 Semiquantitative comparison of adverse event profiles for conventional doxorubicin, and PLD, on a scale from none (–) to severe (+++)*

	Doxorubicin	PLD
Vesicant effect	+++	+/–
Infusion reaction	–	+
Nausea/Vomiting	+	+/–
Myelosuppression	+++	+
Stomatitis/Mucositis	++	++
Hand-foot syndrome	–	++
Cardiotoxicity	+++	+/–
Alopecia	+++	+
Typical dose	60–75 mg/m^2	40–50 mg/m^2
Dose intensity	20–25 mg/m^2/week	10–12.5 mg/m^2/week
Maximal cumulative dose	450 mg/m^2	≥1350 mg/m^2†

*Adapted from Alberts et al.[148]
†Based on current clinical experience, maximal cumulative dose for PLD is at least three times greater than for free doxorubicin.
PLD, pegylated liposomal doxorubicin.

and, given its mild myelosuppressive effect, it was swiftly approved by the FDA in November 1995. The antitumour effect of PLD was obtained as single agent therapy and at a relatively low dose (10–20 mg/m^2) with minimal toxicity. This is probably due to the increased permeability of KS lesions, resulting in high tumour deposition of PLD through the EPR effect[63] and enabling high efficacy at low doses.

PLD has a main role in the treatment strategy of recurrent ovarian cancer. Its regulatory approval in 2001 was based on the results of a phase III study demonstrating prolonged survival with PLD compared with topotecan, particularly in platinum-sensitive ovarian cancer with a 30% risk reduction of death and an 11-month prolongation of median survival.[64 65] A later study, restricted to platinum-sensitive recurrent ovarian cancer, tested a six-cycle regime of carboplatin combined with PLD, against carboplatin with paclitaxel (Calypso study),[66] and demonstrated a statistically superior progression-free survival (PFS) for the former. However, the extended follow-up showed no survival advantage,[67] and cooled off the enthusiasm of the gyneco-oncology community for PLD, despite the fact that any survival advantage was probably blunted by the protocol limitation of six cycles of treatment and by poststudy cross-treatment with PLD in 68% of the carboplatin-paclitaxel arm patients.

In first-line chemotherapy of ovarian cancer, the Multicentre Italian Trials in Ovarian Cancer MITO-2 study tested carboplatin combined with PLD against carboplatin with paclitaxel given for six cycles every 3 weeks.[68] Differences in PFS and overall survival (OS) were not significant. Toxicity-wise, replacing paclitaxel with PLD resulted in a drastic reduction of neurotoxicity and alopecia. Similar results were obtained in a randomised phase II study of the Hellenic Cooperative Oncology Group.[69]

Current guidelines for advanced-stage epithelial ovarian cancer recommend appropriate surgical debulking plus adjuvant systemic chemotherapy in most patients. Neoadjuvant chemotherapy can also be considered for patients who are poor surgical candidates, such as those with poor performance score or bulky disease, in the hopes that tumour load reduction may improve their condition and thereby reduce perioperative risks. In the absence of data indicating that specific regimens should be excluded or favoured, the US national comprehensive cancer network (NCCN) guidelines provide a list of options that can be used before and/or after surgery in patients, including platinum agents, taxanes and PLD.[70] Although primary presurgical treatment is probably the best approach to exploit the EPR effect of PLD for efficacy, no randomised neoadjuvant studies with PLD-based chemotherapy have been conducted or published.

A meta-analysis of the role of PLD in ovarian cancer concluded that PLD is as efficacious as other monotherapies and has better tolerability. In doublets with carboplatin, there is a trend for longer PFS but OS is not improved.[71] As with platinum agents, BRCA1-mutated and BRCA2-mutated ovarian cancer are also particularly sensitive to PLD.[72] In a comparative phase IIB study, the PFS of PLD in BRCA-mutated, platinum-resistant, recurrent ovarian cancer was similar to that of olaparib, a PARP-inhibitor, suggesting that combined therapy could be an attractive approach.[73] While PLD has become an essential tool in the treatment of ovarian cancer, its use is still mainly limited to platinum-resistant recurrent ovarian cancer, thus underexploiting its potential added value.

In breast cancer, the landmark study of O'Brien *et al*[74] established the non-inferiority of PLD versus free doxorubicin in metastatic breast cancer along with a major (~threefold) risk reduction of cardiotoxicity (figure 4C) and led to regulatory approval by the European Medicines Agency in 2003. The cardiac safety profile of PLD enables prolonged treatment and the possibility to combine anthracycline therapy with anti HER2 antibodies. Other head-to-head phase III studies in metastatic breast cancer studies showed modest achievements of single agent PLD compared with other chemotherapies such as mitomycin-c+vinblastine, vinorelbine and capecitabine, confirming non-inferiority but with no indication of superiority in major parameters of efficacy.[75 76] In another phase III study, a combination of PLD and docetaxel was compared with docetaxel monotherapy. The combination arm of the study protocol was problematic resulting in higher rates of skin toxicity and 34% discontinuation rate within the first 3 cycles. The combination had a highly statistically significant improvement in PFS and ORR but lacked survival benefit, which may be due to higher discontinuation rates in the intervention arm.[77] The design of this study ignored the PK interference reported for combinations

of PLD and taxanes, which results in delayed clearance of PLD and consequently severe skin toxicity.[78] If this study would have administered PLD and docetaxel staggered by 1 week along a 4-week cycle, instead of concomitantly every 3 weeks, skin toxicity would have been minimised, and the outcome of the FDA new drug application for PLD in breast cancer could have been positive. Unfortunately, this was not the case and the use of PLD for breast cancer in the USA, as opposed to Europe, remains off label.

The efficacy of PLD versus free doxorubicin in advanced soft tissue sarcoma (STS) was studied by Judson *et al* in a comparative phase IIB trial of the European Organisation for Research and Treatment of Cancer (EORTC) with 94 eligible patients. PLD had equivalent activity to doxorubicin in STS with an improved toxicity profile.[79] A follow-up phase I study[80] with PLD, 30 mg/m^2, and ifosfamide every 3 weeks in advanced STS showed this combination to be feasible, allowing ifosfamide to be given in a dosage similar to that used when given alone. Despite these encouraging data and several other reports of activity in STS, PLD has not replaced free doxorubicin in STS and is seldom used in this indication.

PLD is also an alternative to conventional doxorubicin in the treatment of multiple myeloma where studies have shown equivalent efficacy and reduced toxicity.[76] A randomised phase III study compared monotherapy with bortezomib with combined therapy of bortezomib plus PLD[81] demonstrating improvement of time to disease progression but no significant prolongation of overall survival. These findings established this combination as one of the acceptable standards of care in relapsed or refractory disease.

The wide adoption of PLD, particularly in the first-line setting, has been hindered by non-medical factors: cost—PLD is several fold more expensive than paclitaxel, the comparator drug in ovarian cancer and even more than free doxorubicin; availability—a dramatic shortage in 2011 affected the routine use of PLD and its clinical development worldwide, and, while in the USA, a generic product (Lipodox, Sun Pharma) became available in 2012, there was no generic formulation of PLD approved in Europe until 2022 when Zolsketil (Accord Healthcare) was approved by the European Medicines Agency; reimbursement—the lack of a robust clinical superiority of PLD weakened the pressure on health care providers for reimbursement, therefore limiting the clinical use of PLD.

THE LAST 20 YEARS OF PLD: MIXED CLINICAL SIGNALS

Due to the extensive use of anthracyclines in the field of breast cancer, we will focus on the clinical testing of PLD in this indication. During the early clinical development of PLD, the expectation was that PLD, given its pharmacological advantage in drug delivery and cardiotoxicity, could replace doxorubicin in all treatment settings: neoadjuvant, adjuvant and metastatic. However, these expectations have been only partially fulfilled mainly due to inconclusive proof of superior efficacy of PLD.

The reduced cardiotoxicity of PLD is very relevant in metastatic patients with previous exposure to anthracyclines, or with other cardiac risk factors. In addition, given that any minimal dose of doxorubicin will cause subclinical cardiac damage,[82] we can also argue for use of PLD in any setting in which anthracycline treatment is desired. Particularly, patients with curative potential who have a long life expectancy should also receive PLD, because a subclinical reduction of the cardiac reserve can make these patients susceptible to succumb at older age to pneumonias or other ailments that require an important cardiac reserve. In addition, free doxorubicin exposure may prevent young patients from sustaining intensive aerobic exercise. While replacing anthracyclines with other agents such as taxanes is an option, it is indisputable that anthracyclines are among the most active chemotherapeutic agents in breast cancer and therefore should not be easily dismissed, especially when a non-cardiotoxic alternative such as PLD is available.

Another important direction is the possibility of integrating PLD in anti-HER2 treatment protocols, since doxorubicin and trastuzumab are highly synergistic but the high risk of cardiotoxicity is prohibitive.[83]

We will now review the results of clinical studies in these various settings (see online supplemental table S2 for a list of phase II–III breast cancer studies with PLD in different clinical settings).

Metastatic breast cancer

As mentioned earlier, O'Brien *et al*[74] demonstrated a major reduction in cardiotoxicity with PLD treatment and lead the pathway to several phase III studies.[75 76] Unfortunately, because of FDA rejection of PLD for metastatic breast cancer due to high toxicity in the Sparano *et al.* trial,[77] expiration of patents, and a shortage of drug supply due to manufacturing issues between the years of 2011 and 2013[https://news.bloomberglaw.com/pharma-and-life-sciences/janssen-executive-says-doxil-shortage-likely-due-to-contract-manufacturer], there was a major decline in clinical studies testing PLD under various protocols. A few of those studies in breast cancer are summarized here.

In the metastatic setting, Leonardi *et al*[84] reported a phase II trial aiming to study the efficacy and toxicity of weekly PLD (10 mg/m^2) and paclitaxel (70 mg/m^2) for untreated metastatic breast cancer in patients with high risk for toxicity. Data were collected between the years 2003 and 2007, with an overall response rate of 64.5% and manageable toxicity. Grade 3–4 side effects recorded were mostly hand and foot syndrome. There were also numerous events of cytopenias and mucositis, but no cardiotoxicity was observed.

Alba *et al*[85] conducted a randomised multicentre phase III trial in metastatic breast cancer to evaluate the role of maintenance therapy with six cycles with PLD after induction chemotherapy consisting of three cycles of

doxorubicin followed by three cycles of docetaxel. 155 patients who responded or were stable after induction chemotherapy were recruited between the years 2004 and 2006 and were randomised to PLD versus observation. PLD significantly improved time to progression by 3.3 months (8.4 vs 5.1 months), but OS was not improved (24.8 vs 22.0 months). Toxicity was mild and manageable with 5% of patients experiencing grade 3/4 non-haematological events, and 12% experiencing grade 3/4 neutropenia. Restricting maintenance to six cycles, despite the great cardiac tolerance to high cumulative doses of PLD, was probably too short a period to expect a significant difference in OS given the dilutional effect of poststudy treatments.

Al-Batran *et al*[86] conducted a pooled analysis of four studies with a total of 935 patients treated with PLD and observed a clinical benefit rate (complete response (CR)+partial response (PR)+stable disease (SD)) of 37% in 274 anthracycline-pretreated patients. They concluded that anthracycline rechallenge using PLD is effective in patients with metastatic breast cancer who have a favourable performance status, regardless of setting, resistance, cumulative dose or time since prior conventional anthracycline therapy.

A multicentre phase II, single arm trial, recruited 45 taxane-pretreated patients with metastatic breast cancer to examine the efficacy and safety of 40 mg/m^2 PLD in combination with cyclophosphamide and 5-fluorouracil.[87] The ORR was 42% with a median PFS of 8.2 months; side effects included mainly grade 3–4 cytopenias and manageable non-haematological adverse events, without cardiotoxicity. The investigators concluded this regimen to be a good treatment option after progression under taxane-based treatment, with a safe toxicity profile.

A Spanish long-term retrospective analysis included over 100 patients receiving a combination treatment of PLD and gemcitabine within the years 2001–2014.[88] Patients had a median of three previous systemic regimes in the metastatic setting. Overall clinical benefit rate from this series was 63% with an objective response in 31% of the patients and stabilisation in 32% of the patients, indicating that PLD in combination with gemcitabine is an effective regimen with an acceptable toxicity profile.

Neoadjuvant treatment of breast cancer

Phase II studies addressing the perioperative setting showed promising results for PLD, both in HER2-positive and HER2-negative tumours. In addition, the importance of avoiding cardiotoxicity, particularly during the peri-operative period, is also of great benefit, to ensure adequate and timely surgery rates. As detailed below, studies have shown good response rates and safety profile, and as shown in the metastatic patients, toxicity correlates with dosing and scheduling of the regimen.

A phase II study published by Gogas *et al*[89] included 35 patients receiving treatment with PLD 35 mg/m^2 in combination with paclitaxel 175 mg/m^2 every 3 weeks for six cycles. Response rate was 71%. Toxicity was primarily hand-foot syndrome (grade 3, 9%), and leucopenia (grade 3, 11%). No cardiac toxicity was observed.

A similar combination but with a different regimen was tested by Rossi *et al*,[90] who administered a biweekly low dose of PLD 15 mg/m^2 plus weekly paclitaxel 80 mg/m^2 in the neoadjuvant setting to 35 operable women. The overall response rate was 74%, most of the patients with PR, and 55% undergoing conservative surgery. The main toxicity was hand and foot syndrome (grade 3, 11%). The combination was concluded to be active in operable and locally advanced breast cancer with a manageable safety profile.

Other studies addressed patients with advanced locoregional disease. For example, Torrisi *et al*[91] published in 2011 a phase II trial including 40 patients who received a regimen of cisplatin 60 mg/m^2, infusional fluorouracil 200 mg/m^2 and PLD 25 mg/m^2. The population of the study included patients with T4 tumours, recurrent inflammatory tumours and major nodal involvement. Clinical response rate was 77.5%. Breast conserving surgery was feasible in 16 patients (41%) but this rate raised up to 62% when excluding patients with T4 tumours who were candidates for mastectomy irrespective of clinical response. Unfortunately, the study did not include long-term follow-up results. Regimen was fairly tolerated, but high grade PPE was reported in 5% of the patients.

Another relevant study was CAPRICE,[92] which assessed patients with high-risk breast cancer (stage II–IIIb) with median age of 73 years and different cardiotoxicity risk factors treated with a regimen of PLD 35 mg/m^2+cyclophosphamide 600 mg/m^2 followed by weekly paclitaxel 80 mg/m^2. This regimen achieved similar rates of pathological CR (pCR) as with conventional anthracycline+taxane combination treatment and doubled the rate of breast conserving surgery. Most common side effects were fatigue and sensorial neuropathy, appearing during weekly infusions of paclitaxel. Even though the majority of serious adverse events were below grade 3, only 52% completed the planned regimen. This may be related to a relatively old patient population with comorbidities.

Li *et al*[93] published a trial performed between 2017 and 2018 with 112 patients under the neoadjuvant protocol of PLD 40 mg/m^2+cyclophosphamide 600 mg/m^2 followed by docetaxel 85 mg/m^2 for four cycles each. The primary end point was pCR, which reached a rate of 19% in all patients and 40% in the subgroup of triple negative breast cancer (TNBC). Decrease in left ventricular ejection fraction (LVEF) was not observed during follow-up. Toxicity included mainly grade 1–2 fatigue, nausea, fever and hand and foot syndrome, which reached grade 3–4 in 14% of patients.

Adjuvant treatment of breast cancer

Despite the safety profile advantage of PLD over doxorubicin, no randomised studies were performed comparing the classical and widely used AC (doxorubicin-cyclophosphamide) protocol with a PLD-cyclophosphamide protocol in the adjuvant setting. The

justification for PLD in the adjuvant setting is based mostly on data from metastatic and neoadjuvant trials. Several of the relevant clinical trials addressing adjuvant therapy also investigate the efficacy in HER2-positive tumours (see 'HER2-positive non-metastatic breast cancer' section).

Dellapasqua *et al*[94] performed a phase II trial in the adjuvant setting of luminal B early breast cancer, with a primary end point of feasibility defined as achieving a relative dose intensity of at least 85% in the eight cycles of treatment with a 20 mg/m^2 biweekly regimen of PLD, along with the recommended endocrine therapy. 63 patients with a median age of 49 years were included in the trial between 2016 and 2018. Most patients (87%) completed the treatment. Disease-free survival of 3 years was 97%, after a median follow-up of 3.9 years. Most common adverse events were PPE (12.2%), fatigue (10.4%) and mucositis (8.5%). Only 13% of patients had grade 3 events.

A large retrospective analysis[95] of 1471 patients comparing PLD with epirubicin-based adjuvant therapy in local breast cancer between 2000 and 2018 included 661 patients receiving PLD. The regimen included PLD given at 35–40 mg/m^2 vs epirubicin 90–100 mg/m^2, in combination with cyclophosphamide and followed by docetaxel or paclitaxel. Median follow-up time was 45.9 months. Adjuvant treatment included anti-HER2 antibody if HER2 expression was positive in the tumour. No significant difference in OS or disease-free survival was demonstrated. Analysis of LVEF and ECG documentation suggested that the PLD-based regimen caused less cardiac toxicity than epirubicin.

HER2-positive non-metastatic breast cancer

Due to the risk of cardiotoxicity under treatment with anti-HER2 drugs, combination with PLD instead of conventional anthracyclines is an appealing option to clinicians.

Torrisi *et al*[96] tested the tumour response rate in 32 patients with locally advanced HER2-positive breast cancer under the peri-operative treatment of PLD 25 mg/m^2, cisplatin and infusional 5-fluorouracil (CCF) plus trastuzumab for a total of eight cycles. A clinical response rate of 94% and a pCR rate of 41% in all patients and 54% in patients with inflammatory tumours were observed. Hand and foot syndrome was mostly limited to grade 1–2, and neutropenia was frequent but limited to grade 3 (25%). However, 11/32 patients were unable to complete the scheduled eight cycles. The combination of PLD, CCF and trastuzumab was concluded to be at least as active as the combination of standard chemotherapy and trastuzumab, without events of cardiotoxicity, and was particularly active in inflammatory breast cancer.

The BACH phase II multinational and randomised trial goal was to examine the safety of a concurrent anthracycline-trastuzumab regimen as adjuvant therapy.[97] Patients were randomised between the standard anthracycline protocol (AC→TH) and a protocol with PLD 35 mg/m^2 replacing adriamycin (A, doxorubicin). The primary end point of the study was cardiac event rate or inability to complete 1 year of anti Her-2 treatment. The cardiac safety analysis of this study suggested that administering trastuzumab concurrent with PLD regimen is feasible and resulted in lower rates of early cardiotoxicity and premature cessation of trastuzumab due to cardiotoxicity, compared with AC→TH. Unfortunately, even though the protocol was initially designed to evaluate relapse-free survival in the two treatment arms, efficacy data were not collected.

A recently published study[98] tested a neoadjuvant regimen of four cycles of PLD 35 mg/m^2 with cyclophosphamide followed by four cycles of nab-paclitaxel (Abraxane) with dual HER2 blockade including trastuzumab and pertuzumab in 95 patients. The study primary end point achieved an impressive rate of 80% pathological complete response with disease control rate (CR+PR+SD) of 99%. Four per cent of patients experienced asymptomatic decline of LVEF to borderline values of 43%–49%. 30% of patients experienced grade 3 adverse events, mostly neutropenia. Hand and foot syndrome was not mentioned as an adverse event, but only 'dry skin', with no grade 3 events. The median follow-up time was relatively short, 11 months at the data cut-off, therefore no long-term cardiac safety and no survival outcome data were available until publication.

HER2-positive metastatic breast cancer

In the metastatic setting of HER2-positive breast cancer, Chia *et al*[99] published in 2006 the results of a phase II trial with 30 patients, 13 of them with earlier exposure to anthracyclines, under the treatment of weekly trastuzumab and PLD 50 mg/m^2 every 4 weeks. Response rate was 52% with another 38% of patients with stable disease. Three patients developed asymptomatic decline in LVEF, all of them with prior exposure to anthracyclines. Rates of grade 3 adverse events were relatively high with 30% of grade 3 hand-foot syndrome and >20% grade 3 neutropenia, this may be correlated with the relatively high dose of PLD (50 mg/m^2) given in combination therapy, even if the schedule is every 4 weeks.

The Eastern Cooperative Oncology Group 3198 study[100] had an interesting design, comparing cardiotoxicity between a HER2-negative population (38 patients) receiving first-line treatment with PLD (30 mg/m^2) and docetaxel (60 mg/m^2) every 3 weeks, versus the addition of weekly trastuzumab in patients with HER2-positive breast cancer (46 patients). Response rates were similar on both arms, ranging from 45% to 47%. The PFS was 10.6–11 months and the OS was 24.6 months for the HER2-negative group and 31.8 months for the HER2-positive group. There was no difference in the incidence of cardiac events between the groups. The trastuzumab arm

had more toxicities, especially higher rates of hand-foot syndrome (grade 3—22% in arm A and 38% in arm B) and higher rate of discontinuation. authors concluded that the PLD-docetaxel combination is an effective regimen, particularly when used in conjunction with anti-HER2 treatment.

In conclusion, incorporating PLD as a standard line of treatment in breast cancer is evidence-based, with the overall clinical data supporting its non-inferiority to other chemotherapeutic regimens and demonstrating a noticeable clinical benefit even after several lines of treatment. Cardiotoxicity has been demonstrated to be minimal in numerous trials. The specific regimen, depending on the intensity of dosage and frequency, affects both haematological and non-haematological toxicity, with a significant impact on quality of life. However, the lack of a clear signal of improved efficacy, along with confounding factors regarding the optimal dose, schedule and combination with other agents, prevent its wide adoption in the various settings of breast cancer therapy.

NANOMEDICINE IN THE ERA OF IMMUNOTHERAPY: CAN PLD OUTPERFORM CONVENTIONAL CHEMOTHERAPY IN COMBINATION WITH IMMUNOTHERAPY?

Immune modulatory activity of free doxorubicin

While doxorubicin was initially discovered as a DNA intercalator and inhibitor of DNA topoisomerase II,[101] it has other mechanisms of action, including free radical formation that leads to apoptosis, induction of ferroptosis through lipid peroxidation and modulation of the tumour immunological milieu through pyroptosis. Pyroptosis is an inflammatory programmed cell death pathway associated with caspase-1 activation and release of the cytokines interleukin (IL)-1 and IL-18.[102] Doxorubicin is now known to cause immunogenic cell death via pyroptosis as well as direct effects on both innate and adaptive immunity.[103 104] Immunogenic cell death is a unique form of cell death where immunostimulatory molecules such as damage-associated molecular patterns, calreticulin, ATP and high-mobility group box 1 are secreted from dead cells facilitating activation of a T cell-dependent immune response towards the tumour cells.[105]

Doxorubicin was reported to enhance immune response against murine neuroblastoma cells by triggering the phagocytosis of dead tumour cells by bone marrow-derived dendritic cells, which subsequently led to activation of CD8α+ cytotoxic T lymphocytes (CTL) and increased production of interferon-γ.[106] Doxorubicin was also found to enhance the elimination of tumour-associated myeloid-derived suppressor cells (MDSC) and increase proliferation and function of CTL and natural killer cells in the spleen, blood and in the tumour bed of 4T1 mammary tumours.[107] Doxorubicin specifically promotes apoptosis of MDSC through induction of reactive oxygen species but it does not have any detectable toxic effect on CTL.[107] Patients with triple-negative breast cancer who were treated with doxorubicin showed an enhancement in T-cell cytotoxicity pathways and upregulation of programmed death-1 (PD-1)/programmed death-ligand 1 (PD-L1). This upregulation is accompanied by JAK-STAT pathway and tumour necrosis factor-α signalling activation. Together, the immune modulatory effects and immunogenic cell death associated with doxorubicin suggest that it could significantly improve the efficacy of immune checkpoint inhibitors and other immunotherapies.

Immune effects due to liposome-encapsulation

There are multiple factors that affect the interactions between the immune system and nanoparticles and these factors are size, aggregation properties, crystallinity, composition, shape and surface charge.[108] The interaction between the immune system and nanoparticles could be either immunostimulation or immunosuppression.[108] The latter can result in promotion of tumour growth by plain, drug-free, liposomes.[109] Liposomes often trigger activation of the complement system and cause the release of pro-inflammatory mediators like C3a, C4a and C5a and opsonins such as C3b/iC3b, which facilitate nanoparticle uptake by phagocytic cells.[110] Moreover, liposomes and other nanoparticles can promote tumour growth possibly due to the release of C5a, a complement anaphylatoxin that is known to promote tumour growth and recruitment of MDSC.[110 111] PEG-modification (pegylation) of liposomes decreases opsonisation and the non-specific clearance by the mononuclear phagocytic system or RES. However, in some patients, pegylation exacerbates the complement anaphylatoxin effect due to the pre-existence of circulating anti-PEG antibodies.[111 112]

The encapsulation of doxorubicin in pegylated liposomes (ie, PLD) enhances its immune modulatory effects by redirecting cellular drug uptake to phagocytic immune cells such as TAM and MDSC. The enhancement of tumour growth and immunosuppressive effect related to drug-free liposomes have not been observed with PLD. On the contrary, PLD significantly reduces the number and functionality of MDSC and TAM resulting in enhanced antitumour immune responses in comparison with free doxorubicin.[113] These effects contribute to a greater and significant reduction in tumour growth compared with free doxorubicin at equivalent dose.[113] These results show that liposomal encapsulation of doxorubicin modifies the drug PK and enhances the antitumour immune responses associated with doxorubicin,[113] as summarised in figure 5, and strongly support the application of PLD as part of combination chemo-immunotherapy regimens.

PLD for chemo-immunotherapy

Both doxorubicin and PLD synergise with immune checkpoint inhibitory antibodies (PD-1, PD-L1 and cytotoxic T-lymphocyte antigen 4 (CTLA-4)) in preventing tumour establishment in immunocompetent mouse models of colorectal cancer.[114] However, in the mice bearing established tumours, PLD demonstrated both

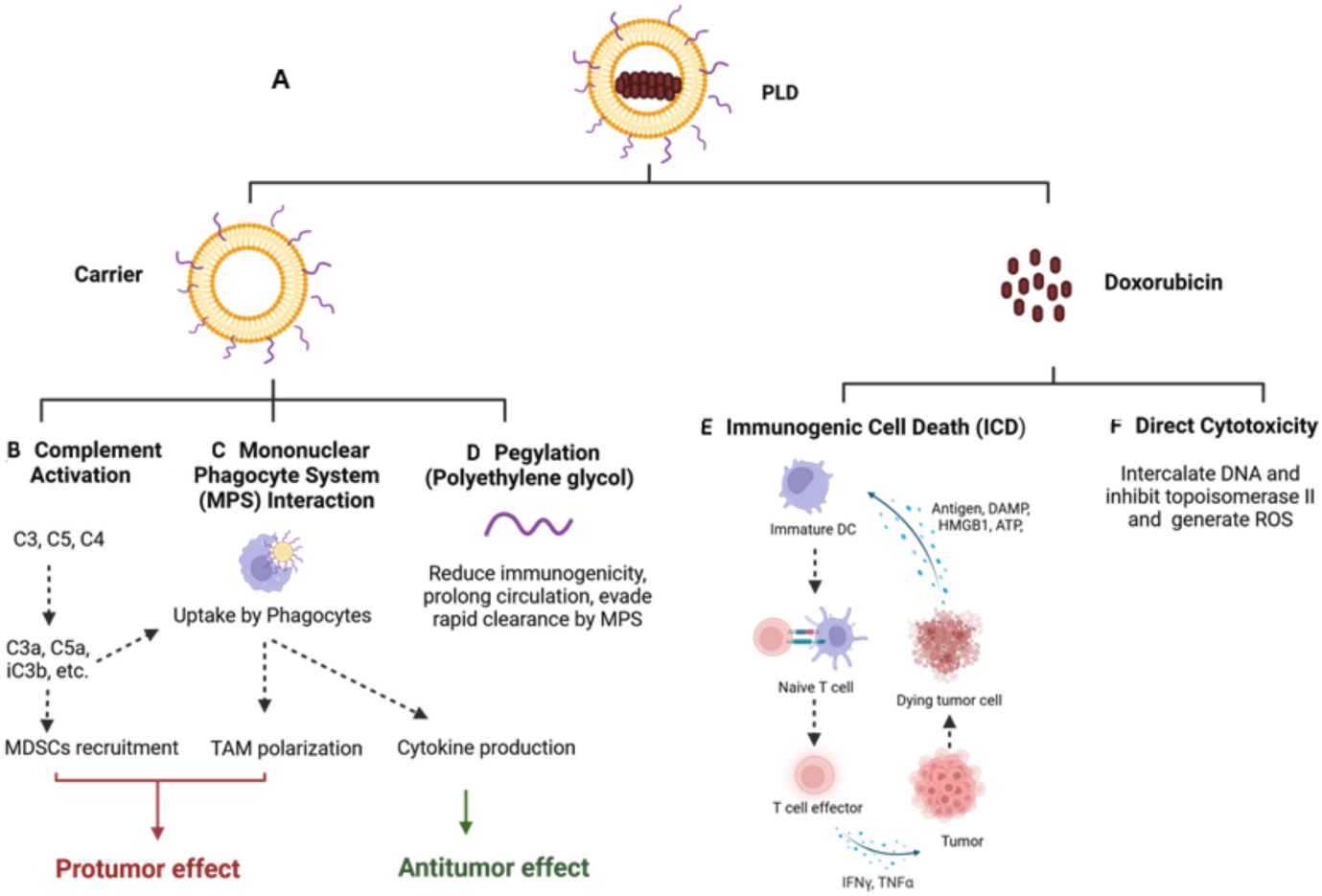

Figure 5 Immunological interactions with Pegylated liposomal doxorubicin (PLD): (A) PLD is composed of a doxorubicin core encapsulated within a pegylated lipid bilayer. There are carrier (B–D) and drug-related effects (E–F). (B) PLD induces complement activation resulting in opsonisation and enhancement of particle clearance. Additionally, complement-derived anaphylatoxins (eg, C5a) can promote tumour growth by recruiting myeloid-derived suppressor cells (MDSCs) to the tumour microenvironment. (C) PLD interacts with the mononuclear phagocyte system (MPS) leading to the production of pro-inflammatory cytokines such as tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and IL-12 that can enhance the antitumour immune response by activation of T cells. On the other hand, PLD decreases M2-polarised tumour-associated macrophages (TAM), which release anti-inflammatory cytokines like IL-10 and transforming growth factor (TGF)-β and can result in immunosuppression and promote tumour progression. (D) Pegylation reduces recognition by the MPS and extends circulation time in blood. (E) Doxorubicin causes immunogenic cell death (ICD) in which dying cancer cells release damage-associated molecular patterns (DAMPs), such as ATP, high-mobility group box 1 (HMGB1) and antigens that result in activation of dendritic cells (DCs) resulting in enhanced antigen presentation and activation of antitumour adaptive immunity. (F) Direct cytotoxic effects of doxorubicin include DNA intercalation which disrupts the topoisomerase II enzyme, and generation of free radicals - reactive oxygen species (ROS).

anticancer efficacy and synergy with immune checkpoint blockade, while doxorubicin did not.[114 115] PLD was active when CT26 tumours were grown in immunocompetent mice but not immunocompromised mice, demonstrating that PLD activity is dependent on the presence of a functional immune system.[114 115] PLD treatment was also associated with enhanced antitumoral immune responses as indicated by diminished tumour infiltration of regulatory T cells and increased CTL in blood and tumours that may explain the synergistic efficacy when combined with immune checkpoint inhibitors.

At the clinical level, one strategy proposed of chemo-immunotherapy is that of priming the immune response with an initial course of immunogenic cell death inducers (particularly cisplatin or doxorubicin) to enhance the sensitivity to PD-1 blockade.[116] Based on our animal studies, priming with PLD should work as well or better. We are using a similar strategy in an ongoing phase II study in patients with metastatic, hormone-refractory, ER-positive breast cancer.[117]

Several clinical studies have been completed or are underway evaluating the use of PLD in chemo-immunotherapy regimens (see online supplemental table S3 for a partial list). The majority of these test PLD in combination with an immune checkpoint inhibitor in patients with solid cancer. The data reported to date show that PLD with an immune checkpoint inhibitor (durvalumab or pembrolizumab) has an acceptable

toxicity profile and promising efficacy in phase I and II trials.[118–120] Disappointingly, the phase III trial results reported thus far with anti-PD-L1 antibodies in patients with ovarian cancer did not show a significant survival or PFS improvement for avelumab in combination with PLD compared with PLD monotherapy[121] or for atezolizumab in combination with bevacizumab and chemotherapy (PLD, paclitaxel) compared with bevacizumab and chemotherapy only [Meeting presentation ASCO 2024 (https://doi.org/10.1200/JCO.2024.42.17_suppl.LBA5501)]. Phase III studies with combos of PLD and anti-PD-1 or anti-CTLA-4 antibodies are still awaited.

Can we make a better 'DOXIL'?

Based on the antitumour activity observed in animal studies with PLD, the first question one may ask is WHY PLD IS NOT MUCH BETTER than free doxorubicin and other free chemotherapeutic agents in clinical studies? One of the factors that can account for this discrepancy is the therapeutic index, which is narrower in humans than in rodents, thus preventing the use of a more effective dose in the clinical setting. The use of body surface for allometric conversion of the PLD dose from mice to humans is probably inadequate for stable nanomedicines that have a distribution volume mostly limited to the intravascular blood compartment. The commonly used dose in humans of 40 mg/m^2 to attenuate mucocutaneous toxicities is the equivalent of ~1 mg/kg PLD, in contrast to dose levels of ~5 mg/kg frequently and safely used in immunocompetent mice. Therefore, Cmax values achieved in humans are substantially lower than in mice which may have an impact on the therapeutic effect. The ability of mice to tolerate greater doses than humans may be related to a faster circulation half-life (threefold to fivefold) and clearance in the former.[49] This may result from a greater mass of the main organs of liposome clearance, liver and spleen, relative to body weight in mice as compared with humans [Liver to body weight: 1:20 in mice, 1:50 in humans]. The importance of the PLD dose for outperforming free doxorubicin is supported by experiments in an animal tumour model, indicating an increasing delta, from 2-fold to 10-fold, in the amount of drug delivered to tumours with increasing doses of PLD are compared with free doxorubicin.[78]

Another factor that can affect the performance of PLD in humans is the EPR effect.[47] We have already indicated that EPR is very variable in human tumours and tends to be lower in metastases. Tumours with an inflammatory component and an abundance of macrophages tend to have high EPR. A huge effort has been taking place in recent years to enhance EPR with various physical and pharmacological approaches. For example, tumour irradiation causes an influx of macrophages at the tumour site that increase nanoparticle deposition in tumours by capturing circulating liposomes and extravasated liposomes in the tumour intersttial fluid, thus preventing their efflux from the tumour bed.[122] In addition, ionising radiation-induced tumour cell death may reduce interstitial fluid pressure (IFP) and decompress tumour blood vessels enabling improved tumour blood flow and better liposome access.

Another way to enhance EPR is with pharmacological agents that can modulate the TME. One example is the work of Stylianopoulos[123] and Stylianopoulos *et al*[124] who proposed mechanical stress as a major barrier to drug delivery and effective cancer therapy and found various agents that could modulate the TME and improve tumour perfusion, an approach that has been referred to as mechanotherapeutics. These agents include anti-inflammatory TGF-β inhibitors (tranilast, pirfenidone), an angiotensin receptor blocker (losartan) and an antihistamine mast cell stabiliser (ketotifen). In this context, we have recently shown in two experimental sarcoma tumour models that ketotifen reduced tumour stiffness, increased perfusion, increased T-cell infiltration and enhanced the therapeutic efficacy of PLD, particularly when given alongside anti-PD-1.[125]

If we focus on the physical characteristics of PLD liposomes, reduction of vesicle size is one obvious approach to enhance tumour drug delivery. PLD is a liposomal suspension of doxorubicin loaded vesicles of ~85 nm average diameter. If we achieve a 50% reduction in diameter, this will result in an eightfold reduction in volume for spheroidal vesicles. This is likely to reduce the hindrance to liposome extravasation and diffusion across the tumour interstitial fluid, although it should be noted that a greater number of particles will be required to deliver the same dose of drug. Talidox (Innomedica, Bern, Switzerland) is a PLD-like formulation with a smaller vesicle diameter and lower drug-to-lipid ratio. It has completed a dose escalation and PK phase I study, demonstrating a good safety profile and a recommended phase II dose of 40 mg/m^2. The circulation half-life of Talidox (~95 hours) is longer than that of PLD (~70 hours) probably because of RES saturation due to a higher number of infused nanoparticles. Interestingly, this did not seem to have a detrimental effect on safety. This long circulation time of Talidox is likely to enhance liposome accumulation in tumours since both observations are directly correlated as mentioned in the section "Stealth liposomes: the pharmacological value in cancer of long-circulating, stable, liposomal drug carriers". Phase II studies of Talidox are on course.

In addition to EPR passive targeting, efforts have been made by multiple investigators to achieve active targeting by adding ligands to liposomes that bind to overexpressed cancer cell receptors. The rationale is that drug delivery to cancer cells will be enhanced by ligand-mediated intracellular delivery of the liposome cargo. Ligand-directed targeting may also reduce further the toxicity of liposomal drugs. Our approach to active targeting was based on the folate receptor, which is overexpressed in many cancer types.[126] The folate receptor targeting approach has resulted in the recent clinical approval of an anti-folate receptor-α antibody and microtubule inhibitor conjugate.[127] We examined the in vitro and in vivo activity

of PLD postmodified by grafting a folate conjugate of PEG-distearoyl phophatidyl-ethanolamine (DSPE) into the lipid bilayer. While the in vitro uptake and cytotoxicity were clearly enhanced when folate-targeted PLD was compared with unmodified PLD, the in vivo therapeutic gain by the intravenous systemic route was marginal although significant.[128] PK-BD studies indicated that folate-targeted PLD was cleared faster than PLD and tumour levels were equivalent or lower, probably accounting for the minimal in vivo therapeutic gain.[129 130] One setting in which folate-targeted PLD was clearly superior to PLD was an intracavitary tumour model of an ascitic tumour treated by intraperitoneal treatment.[128 131] Unfortunately, the potential clinical added value of folate-targeted PLD has never been examined.

At the clinical level, the most advanced project in active targeting of PLD was MM-302, a product of Merrimack Pharmaceuticals (Cambridge, Massachusetts, USA) in which an anti-HER2 single chain Fv was coupled to PEG-DSPE and grafted in the lipid bilayer of PLD. This product had a strong preclinical data package and encouraging phase I data.[132] However, MM-302 in combination with trastuzumab failed to improve the therapeutic end points in HER2-positive metastatic breast cancer in a randomised phase II study (Hermione) against a combo of standard chemotherapy and trastuzumab [https://www.targetedonc.com/view/mm302-misses-endpoint-in-phase-ii-her2-breast-cancer-trial]. While the addition of trastuzumab to MM-302 in the Hermione study[133] conceivably may have hindered the activity of MM-302, it is evident that ligand-mediated targeting of liposomal drugs lags far behind the successful application of antibody-drug conjugates.[134]

Combination therapy protocols with agents given alongside PLD, such as platinum derivatives and other chemotherapeutics, mechanomodulators and immunomodulators as discussed previously, is clearly one more way to improve outcomes. A special combination route and attractive strategy is the co-encapsulation of two APIs in the same liposome. Co-delivery of drugs could optimise in vivo synergistic effects by conferring to both drugs the same PK and BD profiles,[135] particularly when choosing drugs with different mechanisms of action and non-overlapping toxicities. Co-encapsulation of cytarabine and daunorubicin (Vyxeos) at a specific drug-to-drug ratio has demonstrated improved survival of patients with acute myeloid leukaemia when compared with standard treatment and has been approved by the FDA for clinical use.[136] To improve the performance of PLD, we have replaced the non-active ingredient ammonium sulfate with ammonium alendronate, an aminobisphosphonate, and developed a formulation that co-encapsulates alendronate and doxorubicin. This results in the formation of a complex salt that precipitates as rods within spherical vesicles.[137] Owing to the immune modulatory effects of alendronate, pegylated liposomal alendronate of doxorubicin, abbreviated as PLAD, demonstrates unique properties and superior activity when compared with the standard formulation of PLD in immunocompetent mouse models.[125 138]

Another factor to consider in the PLD formulation is the role of the PEG lipid conjugate, which is critical for long circulation time and, thereby, for tumour drug delivery. As mentioned in the section "Immune effects due to liposome-encapsulation", pegylation forms a hydrophilic coating that helps stabilise small vesicles and protect them from opsonisation, thus preventing liposome recognition and fast removal from circulation by phagocytic cells.[139] However, some patients have anti-PEG antibodies that account for a significant fraction of the infusion reactions involving complement activation.[112 140] These infusion reactions, triggered by multiple factors, can be life threatening and mandate the use of premedication and a very low start drip rate of PLD (1/4 of less of the target drip rate) with cautious stepwise increases, at least during the first course of PLD. Fortunately, experimental studies have shown that PLD suppresses B cells producing these antibodies[141] and prevents in most cases reactions in subsequent cycles of treatment. Yet, we cannot rule out the frequent occurrence of complement activation at subclinical levels. As reviewed by La-Beck *et al*,[23] complement activation can cause accelerated liposome clearance and trigger an inflammatory reaction, which can have a detrimental effect on liposome passive targeting to tumours and promote tumour growth. Complement attack may even lead to drug leakage, particularly in the poles of elliptic liposomes.[112 142] Inhibition of acute complement responses with co-administered complement regulator constructs has been shown to effectively block complement opsonisation and accelerated clearance of nanoparticles.[143] In vitro tests to detect patients at increased risk for acute infusion reactions to pegylated liposomes have been proposed.[144] Furthermore, some changes in the formulation of PLD can reduce the risk of complement activation: (i) removing the negative charge of the phosphate group of PEG-DSPE conjugate to avoid interaction with calcium cations by replacing it with a neutral conjugate such as PEG-distearoyl-rac-glycerol (DSG); (ii) making smaller liposomes as in the case of Talidox; (iii) maintaining a spherical shape and avoiding formation of elliptic (ovoid) liposomes by reducing the drug-to-lipid ratio (Talidox) or replacing sulfate with another counter-anion as in the case of PLAD.

In conclusion, the potential contribution of PLD to cancer therapy can be exploited further using rational combinations and multimodality-based therapy for synergistic effects, and empowering immunotherapy. Further optimisation of the physico-chemical properties (size, shape, surface charge) of PLD, particularly when combined with the above strategies, is also a worthy approach to improve the translation success of PLD and other cancer nanomedicines.

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contributor to the conception and design of the review, as well as the analysis and/or interpretation of data for the review. All authors gave their final approval of the version to be published. AAG is accountable for all aspects of the work, ensuring that questions related to the accuracy or integrity of any part of the review were appropriately investigated and resolved. AAG is responsible for the overall content as the guarantor.

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