Graphic Abstract

The success of combination chemotherapy requires precise delivery of synergistic drugs to cancer cells while sparing normal tissues. Here we demonstrated that co-encapsulating doxorubicin and mitomycin C within polymer-lipid hybrid nanoparticles (DMPLN) altered their biodistribution, pharmacokinetics, and toxicity profiles. DMPLN prolonged systemic circulation, increased accumulation, and maintained synergistic molar ratios of doxorubicin and mitomycin C within tumors, leading to significantly enhanced antitumor efficacy as compared to co-administrated free drugs. DMPLN also dramatically reduced toxic metabolite of doxorubicin and drug associated organ toxicity.

Polymer-Lipid Hybrid Nanoparticles Synchronize Pharmacokinetics of Co-encapsulated Doxorubicin-Mitomycin C and Enable Their Spatiotemporal Co-delivery and Local Bioavailability in Breast Tumor

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Abstract

Effective combination chemotherapy requires the delivery of drugs of synergistic activities to tumor sites while sparing normal tissues. Herein we investigated whether co-encapsulation of doxorubicin and mitomycin C within polymer-lipid hybrid nanoparticles (DMPLN) achieved this goal *via* ratiometric drugs in an orthotopic murine breast tumor model with nanocarrier-modified biodistribution, pharmacokinetics, local bioavailability and toxicity. Fluorescence imaging revealed quickened and extended tumor uptake of DMPLN but reduced cardiac accumulation. Quantitative drug analysis demonstrated prolonged systemic circulation, increased tumor accumulation and sustained synergistic ratios of doxorubicin and mitomycin C delivered by DMPLN over 24 h. Higher levels of tumor cell apoptosis and reduced organ toxicity were obtained with DMPLN compared to free drug cocktails. DMPLN efficiently released DOX in tumors than that from liposomal doxorubicin, as evidenced by a higher extent of the metabolite, doxorubicinol. These findings substantiate the importance of rational design of nanoparticles for synergistic drug combination therapy.

Keywords: biodistribution, pharmacokinetics, polymer-lipid nanoparticles, doxorubicin combination, ratiometric delivery

Abbreviations

AKR - Aldo-keto reductase

AUC – Area under the curve

CL – Total body clearance

cTnI – Cardiac troponin I

DMPLN – Doxorubicin and mitomycin C co-loaded polymer-lipid hybrid nanoparticles

DOX - Doxorubicin

DOXOL – Doxorubicinol

EPR – Enhanced permeability and retention

HPESO - Hydrolyzed polymer of epoxidized soybean oil

HPLC – High-pressure liquid chromatography

ICG - Indocyanine green

ICG-DMPLN - Indocyanine green coloaded doxorubicin and mitomycin C polymer-lipid hybrid nanoparticles

i.v. – Intravenous

k – Micro rate constant

LC-MS/MS – Liquid chromatography-dual mass spectrometry

MMC - Mitomycin C

NIR – Near infrared

PDI – Polydispersity index

PEG – Polyethylene glycol

PLD – Pegylated liposomal doxorubicin

PLN – Polymer lipid nanoparticles

PK – Pharmacokinetics

$t_{1/2}$ – Half-life

V_{ss} – Volume of distribution at steady state

Background

Drug combination regimens are frequently used in the clinic to enhance cancer chemotherapy¹⁻³. Meta-data analyses of multicenter clinical trials indicate that combination chemotherapy (i.e., two or more drugs administrated simultaneously or on the same day) produces a higher tumor response rate than a single agent or sequential single anticancer drug combination for the aggressive treatment of advanced and metastatic breast cancer^{4,5}. However, owing to increased incidences of adverse effects, the simultaneous administration of drugs in combination is not recommended by current chemotherapy guidelines in which the maximum tolerated dose is applied for each individual drug⁶⁻⁸. Nonetheless, Phase II clinical trials using low dose metronomic drug combinations and varying schedules have recently showed a greater positive tumor response and lower toxicity profiles^{9,10}. These different results could be ascribed to differences in the spatial and temporal delivery of drug combinations to the tumor as well as differences in peak drug doses and drug exposure of normal tissue.

Theoretically, anticancer drugs showing synergistic cytotoxic effects on cancer cells after simultaneous exposure *in vitro* should generate higher anticancer efficacy *in vivo* than the sequential administration of individual drugs, if each would reach cancer cells simultaneously in an optimal ratio. Unfortunately, dissimilar pharmacokinetics (PK) and tissue disposition of different drugs given in free solutions could preclude their co-existence and co-action in cancer cells for optimal killing, thus compromising the therapeutic effectiveness of combination chemotherapy¹¹. Moreover, co-administration of multiple drugs in free solution at normal therapeutic doses often results in increased normal tissue toxicity.

To mitigate these problems encountered by free drug combination chemotherapy, nanoparticles have been formulated to co-load synergistic drug combinations ¹¹⁻²⁰. Well-designed nanoparticles can alter the PK and toxicity profiles of the free drug combination, enable drug accumulation in the tumor and release the drugs at a synchronized rate, thereby maintaining synergistic drug ratios to achieve enhanced anticancer effects. Several liposomal formulations have demonstrated the capability of delivering drugs at synergistic ratios to tumor tissues resulting in elevated drug efficacy in mice ^{15, 20}. Furthermore a liposomal formulation of irinotecan and floxuridine at 1:1 molar ratio, shows enhanced therapeutic efficacy and safety in a phase I clinical trial on patients with advanced solid tumors ¹², and is now moved into a Phase II trial ¹³.

The successful development of nanoparticle-based drug combinations also depends on the rational selection of drug candidates. A basic consideration is that the individual drugs should be clinically effective for treatment of the cancer of interest. Moreover, several other factors have been considered, e.g. non-overlapping drug toxicity, non-cross drug resistance mechanism, different mechanisms of drug action or pathways, and synergistic drug-drug interactions ¹⁶⁻²². We had previously investigated the combination of doxorubicin (DOX) and mitomycin C (MMC) ^{21, 23, 24}, in which DOX is used as first line chemotherapy for many solid tumors but is ineffective for quiescent cells ^{25, 26}, whereas MMC exerts cytotoxicity against non-proliferating cells in hypoxic tumor regions *via* enhanced bioreductive activation ²⁷. Through systematic *in vitro* studies of scheduling and dosing of free drug combinations, we discovered that DOX and MMC could generate synergistic cytotoxicity in breast cancer cells when given together, or when DOX was administered earlier than MMC ^{21, 23, 24}. Therefore, we developed DOX-MMC co-loaded polymer-lipid hybrid nanoparticles (DMPLN) and demonstrated their ability to overcome

membrane-efflux transporter-mediated multidrug resistance with much greater potency than the co-administered, single-drug loaded PLN^{17, 19}. The DMPLN exhibited superior anticancer efficacy to PEGylated liposomal DOX (PLD, Caelyx[®]) and minimal systemic and cardiotoxicity in preclinical models of murine and human breast tumor^{18, 19}. These encouraging results prompted us to further investigate the underlying mechanisms that contribute to the superior *in vivo* performance of the DMPLN formulation. We hypothesized that synchronization of DOX-MMC release and spatiotemporal co-delivery of both drugs to cancer cells are crucial for obtaining such superior therapeutic outcomes. Therefore we designed this study to determine biodistribution of the nanocarrier, pharmacokinetics, tumoral and cardio exposure of the drugs in various formulations, and to find the link between physiological changes to drug formulations (pharmacokinetics) and body response to drug formulations (pharmacodynamics: efficacy and safety). We conducted a preclinical evaluation of the biodistribution and PK of DOX and MMC delivered by PLN compared with free drugs and PLD (Caelyx[®]) in mice bearing orthotopic EMT6 breast tumors that are known to contain hypoxic regions^{28, 29}. Acute organ toxicity was examined by histopathology and cardiotoxicity specifically by the presence of plasma cardiac troponin I (cTnI)³⁰. Intratumoral drug concentrations and tumor cell apoptosis were determined as a measure of local drug bioavailability and efficacy respectively. A thorough understanding of the mechanism is of great importance for rationale design of nanoparticle-based chemotherapy regimens.

Methods

Preparation and characterization of PLN

DMPLN was prepared according to Shuhendler *et al.*, 2014¹⁹ and indocyanine green (ICG) co-loaded DMPLN (ICG-DMPLN) was formulated with modifications. To facilitate DOX hydrochloride partition into the lipid phase, an anionic hydrolyzed polymer of epoxidized

soybean oil (HPESO) was used to neutralize cationic charge of DOX³¹. Particle size and zeta potential of the nanoparticles were measured by Zetasizer (Nano-ZS, Malvern, England). The loading content and loading efficiency were determined spectrophotometrically following centrifugation using a filter with 0.1µm pore size³¹. The amounts of DOX and MMC released from the DMPLN into phosphate buffer saline (pH = 7.4) were assayed. The detailed formulations and procedures are presented in Supplemental Materials.

Orthotopic breast tumor model

All animal experiments were approved by the Animal Care Committee of University Health Network at the Ontario Cancer Institute and conducted in accordance with the Canadian Council on Animal Care Guidelines. All mice had free access to food (irradiated Teklad LM485, Harland Teklad, Indianapolis, IN, USA) and sterile water. Orthotopic breast tumors were inoculated by injection of 1×10^6 EMT6/WT murine breast cancer cells into the right mammary fat pads. Mice were used for studies once the tumor size reached $150 \text{ mm}^3 - 200 \text{ mm}^3$ (Supplementary Materials-Table 3S).

Determination of DMPLN biodistribution

The biodistribution of DMPLN was examined by *in vivo* whole body and *ex vivo* organ fluorescence imaging using loaded ICG as a fluorophore. One hundred and eighty µL of ICG-DMPLN containing ICG dose of 1.2 mg/kg was injected intravenously (*i.v*) via the tail vein of tumor-bearing mice. At 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h, mice were anaesthetized with 2% isoflurane via nose cones and *in vivo* whole body images were acquired using a Xenogen IVIS Spectrum (Caliper Life Sciences, Inc. Hopkinton, MA, USA) imager at excitation (λ_{ex}) and emission (λ_{em}) wavelengths of 745 nm and 840 nm, respectively. For *ex vivo* imaging, mice

were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (13 mg/kg), whole blood collected from the posterior *vena cava*, followed by organ perfusion using ice-cold saline *via* the left ventricle. Major organs (heart, lungs, liver, spleen, intestine, and kidneys) and breast tumors were dissected and imaged. To verify that free ICG was eliminated quickly, as reported in the literature ³², in our breast tumor-bearing mice, free ICG dissolved in 5% sterilized dextrose was also injected in separate mice.

Pharmacokinetics and biodistribution of DOX and MMC from various formulations

Tumor-bearing mice were randomly allocated into four treatment groups as follows: 1) free DOX; 2) PLD (Caelyx[®]); 3) free DOX-MMC; 4) DMPLN, at a DOX dose of 9.2 mg/kg, equivalent to a human clinical dose at 25 mg/m² ^{33, 34} with an additional MMC dose of 2.9 mg/kg for DMPLN and free DOX-MMC treatments (Supplementary Materials-Table 3S). At designated time points, mice were anesthetized prior to withdrawal of whole blood and retrieval of organs and tumors after perfusion with ice-cold saline. The organs and tumors were weighed, frozen immediately in liquid nitrogen, transferred into 1.5 mL polypropylene tubes and stored at -80°C. Whole blood was stored in sodium heparin coated plastic tubes (BD, Mississauga, ON, CA) at -20°C until analysis. After extraction from biological samples, DOX, DOXOL and MMC were assayed by high pressure liquid chromatography (HPLC) or liquid chromatography-dual mass spectroscopy (LC-MS/MS). Detailed methods for drug extraction and analysis by HPLC and LC-MS/MS are described in Supplementary Materials (Figure 3S & Figure 4S).

For comparison of the pharmacokinetic parameters of DOX in various formulations and MMC for DMPLN, blood concentration-time profiles were fitted to the two compartment model to obtain the micro-rate constants (k_{10} , k_{21} , k_{12}) with the program *ADAPT5*[®] (Biomedical

Simulation Resource, University of Southern California, Los Angeles, CA, USA) using the following rate equations:

$$\text{Central compartment: } \frac{dC_1}{dt} = \frac{(k_{12} \times A_2 - (k_{10} + k_{12}) \times C_1)}{V_1} \quad \text{Equation 1}$$

$$\text{Peripheral compartment: } \frac{dA_2}{dt} = k_{12} \times V_1 \times C_1 + k_{21} \times A_2 \quad \text{Equation 2}$$

where C_1 and V_1 are the concentration and volume of drug in central compartment, respectively, and A_2 is amount of drug in peripheral compartment; k_{10} is the elimination rate constant from the central compartment; k_{12} is the transfer rate constant from the central to peripheral compartment, and k_{21} is the transfer rate constant from the peripheral to central compartment (Supplementary Materials-Table 4S). V_1 was obtained as the $\text{Dose}_{i.v.}/(A+B)$ where A and B were the y-intercepts of the bi-exponential equation, $C = Ae^{-\alpha t} + Be^{-\beta t}$. The area under the curve from time 0 to infinity ($\text{AUC}_{0-\infty}$) was used to estimate the total clearance ($\text{CL} = \text{Dose}_{i.v.} / \text{AUC}_{i.v.}$), and the volume distribution at steady state (V_{ss}) was estimated as $V_{ss} = V_1 \times (1 + k_{12}/k_{21})$. Data of MMC in free DOX-MMC solution were best fitted to the one compartmental model using the equation:

$$\text{One compartment: } \frac{dC}{dt} = -k \times C_1 \quad \text{Equation 3}$$

V_1 was obtained as the $\text{Dose}_{i.v.}/C_0$ where C_0 was the y-intercept of mono-exponential equation, $C = C_0 e^{-kt}$.

Evaluation of acute toxicity caused by various drug formulations

To examine acute drug toxicity, a DOX dose of 17 mg/kg in mice was used. This dose was higher than that employed for the pharmacokinetics study, but is equivalent to the clinical

human dose for PLD (Caelyx[®]) that is devoid of prominent acute toxicity in the treatment of breast cancer^{33, 35}. DOX delivered by nanoparticles has been compared with free DOX at the same dosage (15-20 mg/kg) for short-term toxicity studies in *Balb/c* mice. For the DOX-MMC combination, 5.8 mg/kg MMC was included to investigate whether DMPLN was capable of reducing adverse effects at the high dose compared to free DOX and free DOX-MMC treatments. Breast tumor-bearing mice were randomly divided into 5 groups with 4 mice per group: 1) free DOX; 2) PLD (Caelyx[®]); 3) free DOX-MMC; 4) DMPLN; and 5) saline (control). Body weights were measured the day before the treatment daily and for 5 days post-treatment (the end-point for all mice groups). Blood was collected into heparin-coated tubes (BD), then immediately centrifuged at 2,000 g for 10 min at 4°C to isolate plasma (Eppendorf 5702R, Eppendorf Corp. Mississauga, ON, CA). Plasma was assayed for cTnI levels using the mouse cardiac troponin I ELISA kit (Life Diagnostics, Inc. West Chester, PA, USA).

For histopathological evaluation of organ toxicity, the heart, liver, and kidney were collected, fixed in 10% formalin and stained with haematoxylin and eosin (H&E) stain. The severity of cardiac, hepatic, and renal lesions was assessed semi-quantitatively by light microscopic examination. The findings were graded using the following scale: no changes (0), minimal (1), mild (2), moderate (3), and marked (4). The median score was used as an estimate of the overall organ toxicity.

Evaluation of drug induced apoptosis in breast tumors

At 24 and 48 h post-treatment, mice were euthanized and tumor xenografts were removed and immediately fixed in 10% neutral buffered formalin for 72 h at room temperature. Tumors

were cut into slices, processed into paraffin blocks, sectioned into 5 μm -thick sections, and then analyzed for apoptosis using immunohistochemical staining (Supplementary Materials).

Statistical Analysis

Results are presented as mean $\pm$ standard error of mean (SEM) of at least three repeats. Statistical significance between different groups was determined by *one-way ANOVA* following the Holm-Sidak method with *p* values <0.05 considered significant (GraphPad PRISM[®] version 6, San Diego, CA, USA).

Results

Particle properties and release kinetics of DMPLN and ICG-DMPLN

Figure 1A and 1B show characterization of both DMPLN and ICG-DMPLN with the particle size hovering around 150 nm, zeta potential of approximately -25 mV, and the polydispersity indices (PDI) about 0.37. Encapsulation efficiency of DOX was greater than 90% in both PLN formulations and MMC encapsulation efficiency was close to 50% in DMPLN and 43% in ICG-DMPLN formulations (Figure 1A). The DMPLN demonstrated sustained release profiles of DOX and MMC over 72 h, whereas free DOX-MMC was completely released from the dialysis tube into the PBS medium within 2 h (Figure 1C). At early time (0-3 h), DOX was released faster than MMC, then both drugs exhibited similar release rate at later times (4-72 h).

Biodistribution of DMPLN

Figure 2A presents the whole body biodistribution of ICG-DMPLN at baseline and at predetermined time points over 24 h by non-invasive fluorescence imaging after a single *i.v.* injection. ICG-DMPLN distributed throughout the whole body immediately after injection. At

0.25 h post-injection, ICG-DMPLN accumulated in the liver and the right mammary fat pad, where the breast tumor was located. The fluorescence intensity in the liver area quickly reduced and the signal shifted to the gastrointestinal region. By 24 h, PLN was mostly eliminated (some through feces, data not shown).

The major organ distribution and breast tumor accumulation of ICG-DMPLN and free ICG were also evaluated by *ex vivo* imaging. As expected, free ICG was eliminated from the body rapidly and did not show any tumor accumulation at 0.25 h post injection (Figure 2B). In contrast, ICG-DMPLN was retained in most organs except the heart and accumulated in the tumor from 0.25 h onward. Since free ICG was quickly eliminated³² and since ICG was not released from the ICG-DMPLN for up to 24 h (Supplementary Material-Figure 1S), the biodistribution of the fluorescent signal in ICG-DMPLN treated mice can be viewed as that for the intact DMPLN.

The relative concentration of DMPLN in whole blood, tumor, and various organs as a function of time was quantified by calculating the relative intensity of ICG-DMPLN-treated samples to that of untreated samples. As depicted in Figure 3 A-H, DMPLN exhibited the highest accumulation in the intestines, followed by the liver at early time points with much weaker fluorescent signals in the heart and the spleen, which is in agreement with the whole body images (Figure 2A). Retention of DMPLN in tumors lasted at least 24 h (Figure 3B), while the signal in the whole blood and major organs diminished by 24 h. The observation is consistent with our previous report on quantum dots in a similar lipid nanoparticle formulation³⁶; the highest fluorescence intensity within the EMT6 breast tumor occurred 0.25 h after *i.v.* injection,

suggesting the ability of PLN to simultaneously deliver both DOX and MMC at the desired site of action.

DMPLN extended the circulation time of DOX and MMC

The total drug concentration profile in whole blood following a single *i.v.* administration of free DOX-MMC or DMPLN formulation in breast tumor-bearing mice is presented in Figure 4A. For comparison, the PK profiles of free DOX and PLD were also measured (Supplementary Materials-Figure 4S).

Table 1 summarizes the PK parameters of both DOX and MMC delivered by various formulations, fitted by the ADAPT5 PK modeling program. The concentrations of DOX and MMC delivered by DMPLN were decreased bi-exponentially over 24 h with a long elimination phase. The DMPLN that entrapped the MMC altered the PK profile of MMC from a one-compartment model to that of a two-compartment model (Figure 4A) with a significantly extended half-life ~ 36 h compared to ~1 h for MMC in the free DOX-MMC formulation (Table 1). The volume of the central compartment (V_1) of MMC was ten times smaller in DMPLN than that for free DOX-MMC (Table 1). DMPLN increased the blood AUC of DOX and MMC by 185% and 584 % compared to the free DOX-MMC group. Compared to the free DOX-MMC combination, the total body clearance (CL) of DOX and MMC in DMPLN was reduced by approximately 66% and 86% respectively.

DMPLN enabled co-delivery of effective DOX and MMC ratios to the breast tumor

Figure 4B and 4C compare DOX and MMC concentrations in the tumor at different times delivered by various formulations. Apparently, DMPLN delivered much more DOX and MMC than free DOX (2-7 fold) and free DOX-MMC (DOX: 7-43 fold; MMC: 3 fold) to the tumor site and maintained both drugs within the tumor for at least 24 h (Figures 4B-4D). More interestingly, DMPLN maintained similar molar ratios of DOX to MMC at 1.24 to 3.42 (Figure 4E), ratios that were found to exhibit synergistic cytotoxicity against breast cancer cells in our previous work^{16, 19, 24}. In contrast, free DOX-MMC reached the tumor at much lower levels and at a low molar ratio of 0.33 – 0.43 in early time points with no detectable MMC at 24 h (Figure 4E). Owing to its long circulation PK profile and the enhanced permeability retention (EPR) effect³⁷, PLD (Caelyx[®]) showed the highest DOX accumulation in tumor at later time points compared to other formulations. However, as illustrated below, the high tumor concentration is not necessarily translatable to high anticancer efficacy.

DMPLN increased local bioavailability and induced more apoptosis in breast tumors

In breast tumor, free DOX or DOX released from the nanoparticle formulations were converted into the DOX metabolite, DOXOL, whereas the DOX remaining in the nanoparticles or outside cells would not be metabolized. Therefore, the DOXOL concentration within the tumor suggested local bioavailable DOX (i.e., free DOX) within tumor cells. As expected, the percent of DOXOL rapidly formed from accumulated DOX was the highest for free DOX group followed by DMPLN (Figure 5A). Almost 15% of the accumulated DOX was converted to DOXOL as early as 0.5 h and DOXOL formation continued over 24 h in the free DOX-treated tumors (Figure 5A). DMPLN-treated tumors exhibited gradually increasing DOXOL levels with time, implying sustained release of DOX from the DMPLN inside tumors. In contrast, DOXOL

was not detectable in the PLD (Caelyx[®]) treated tumors until 24 h (Figure 5A & 5B). These results suggest that DOX released from PLD entering cancer cells was minimal, which was discrepant from the highest total DOX concentration in the tumor among all studied formulations. Since only free or released DOX can exert its pharmacological effect on cancer cells, the local bioavailability of DOX should be a better predictor of anticancer efficacy than the total amount of DOX in the tumor.

Another biomarker of the presence of bioavailable DOX is the activation of caspase-3. Immunohistochemical staining of *caspase-3* (+) cells revealed that DMPLN induced significantly higher tumor cell apoptosis than other DOX formulations at 24 and 48 h post-treatment (Figure 6A and 6B). Despite the long circulation time and high tumor accumulation, PLD (Caelyx[®]) induced less apoptosis than DMPLN at the same DOX dose (9.2 mg/kg). Besides the combination with MMC, higher local bioavailability of DOX from DMPLN may contribute to this higher apoptosis. By contrast, free DOX and free DOX-MMC caused less apoptosis than DMPLN owing to their lower accumulation and retention in the tumor.

DMPLN reduced DOX and DOXOL levels in the heart

One prominent feature of DMPLN is its ability to minimize accumulation of DOX (Figure 7A), and DOXOL (Figure 7B) in the heart, shown by the lower levels and the shortest retention of DOX and DOXOL among all studied formulations. Free DOX and DOX-MMC formulations showed significantly higher DOX and DOXOL levels in the heart than the nanoparticle formulations in the first 6 h and these were lower than PLD at 24 h. By contrast, no detectable DOX or DOXOL was found in the hearts for the DMPLN treated mice at 24 h. Co-

administration of free DOX formulation was associated with higher conversion of DOX to DOXOL in the heart (Figure 7B).

DMPLN exhibited minimum cardiac, hepatic and renal toxicity

To further confirm a positive correlation between the accumulation of DOX and DOXOL and acute cardiac injury at a biochemical level, the plasma concentration of cTnI, a biomarker of early onset cardiac injury, was measured in mice treated with different DOX containing formulations at dose of 17.2 mg/kg or in combination with 5.8 mg/kg MMC. Figure 8A shows that the free DOX-MMC combination resulted in the highest cTnI concentration among all treatments, with the cTnI concentration being elevated significantly in both free DOX and PLD treatments compared to that of saline and DMPLN ($p<0.05$). By contrast, DMPLN showed a marked and significant protection against DOX induced-cardiomyopathy with cTnI levels being undetectable.

To assess the systemic toxicity, body weights of mice were also monitored after a single *i.v.* administration of each formulation with loss of 20% of total initial body weight as a toxic endpoint. Figure 8B shows the % body weight change post-treatment for each group. The DMPLN and saline groups showed no significant weight loss during the 5 day post-treatment. PLD (Caelyx[®]) caused a mild (4%) reduction in body weight, whereas free DOX and DOX-MMC combination resulted a 9% and an approximately 20% body weight reduction respectively at the 5 day post injection end point.

DMPLN demonstrated minimum toxicities compared to other formulations as indicated by histopathology evaluation of the heart, liver, and kidney toxicity (Figure 8C & Table 2). Both free DOX and free DOX-MMC resulted in moderate to marked cardiomyocyte vacuolation and

renal tubular intraluminal protein casts (Table 2). It is worth noting that both PLD and DMPLN nanoparticle groups resulted in mild periportal hepatocellular hypertrophy with cytoplasmic basophilia, a non-adverse response, which was not observed in the free DOX and free DOX-MMC formulations.

Discussion

Free DOX and MMC exhibit elimination profiles distinctively different from each other. In rodents, DOX is mainly metabolized by the liver and biliary system with a 7-10 h elimination half-life³⁸; whereas MMC is cleared primarily through urinary excretion with a short half-life of 15-40 min³⁹. However, when they are administered concomitantly, drug-drug interactions occur, causing increased volume of distribution of DOX and $t_{1/2}$ of both DOX and MMC (Table 1). The increased volume of distribution could be ascribed to the competition between DOX and MMC for red blood cells and plasma proteins due to their strong binding affinity; the competition and displacement from binding could lead to increased free drug and unbound fractions of the drug^{40,41}. The increased distribution volume could contribute to lower drug bioavailability reaching the tumor site and enhanced adverse effects⁴². By contrast, the co-encapsulation of DOX and MMC into PLN could enable their retention within nanocarriers and extend the circulation times of both drugs, as reflected by increased blood AUC and decreased CL (Table 1). DMPLN accumulates passively in the tumor *via* the EPR effect due to the leaky tumor blood vessels and malfunctioning lymphatic vessels⁴³ leading to increased concentrations of DOX and MMC within the tumor as compared to the free drug treatment.

Previous *in vitro* mechanistic studies of DOX-MMC interactions suggested that bioreductive metabolism of MMC could increase intracellular DOX concentration by depletion

of glutathione and facilitate DNA cross-linking to activate DNA repair enzymes colliding with DOX induced topoisomerase II α inhibition, thus increasing DNA double strand breaks²¹. Moreover, it was previously found that co-application of single agent loaded PLNs (i.e., DOX-PLN plus MMC-PLN) was not as effective as the dually loaded DMPLN evidenced by lesser DNA double strand breaks and cytotoxicity in breast cancer cells in culture¹⁶. Based on these findings and our previous scheduling and dosing studies²⁴, it is surmised that DOX and MMC must be present inside the cell at the same time to fully realize their synergism. Indeed, stable co-encapsulation of DOX-MMC within nanoparticles and synchronized release of both drugs from DMPLN ensure the co-delivery of DOX and MMC into the cancer cells spatially and temporally, thus amplifying the DOX-MMC interactions synergistically.

DOX is metabolized to DOXOL through reduction of the carbonyl group by an enzyme aldo-keto reductase (AKR) in the cytosol⁴⁴. Formation of DOXOL reduces the antitumor activity of the parent drug, DOX, but increases the incidence of cardiotoxicity^{45,46}. High levels of DOXOL observed in the heart treated with free DOX and free DOX-MMC groups are associated with prominent acute cardiotoxicity. The co-treatment of MMC with DOX may increase cardiac AKR expression thus accelerating the DOX-to-DOXOL conversion⁴⁹. But the underlying mechanism requires further investigation.

Compared to free DOX, DMPLN shields DOX from metabolism in the tumor resulting in reduced DOXOL formation. Nonetheless, the sustained release of DOX from DMPLN shown by the increased amount of intratumoral DOXOL with time (Figure 5B) is encouraging from a therapeutic standpoint. In comparison, DOXOL level in tumors treated with PLD (Caelyx[®]) was not detectable until 24 h, probably because PLD (Caelyx[®]) did not release DOX before 24 h due

to its long release half-life of 118 h^{47, 48} or cellular uptake of DOX released from PLD (Caelyx[®]) was negligible⁵⁰, suggesting that DOX might not be bioavailable within cancer cells before 24 h.

Among all the studied formulations, DMPLN induces the highest caspase-3 activation in breast tumors at both 24 h and 48 h post treatment (Figure 8), while PLD (Caelyx[®]) induces a similar extent of cell apoptosis as free DOX, although PLD showed higher DOX accumulation in tumor (Figure 4B) and the longest circulation time (Supplementary Materials-Figure 4S). These findings infer the superior efficacy of DMPLN *in vivo* compared to the observed efficacy of PLD (Caelyx[®])^{18, 19}, and confirm the comparable efficacy of PLD (Caelyx[®]) to free DOX in clinic^{50, 51}. Taken together, the observations suggest that the efficacy of DOX cannot be predicted solely based on the exposure of total DOX levels in plasma or in tumor tissue^{52, 53, 54} and the local bioavailability of DOX released from nanocarriers could play a pivotal role in anticancer efficacy.

In summary, the present study demonstrates, for the first time, that compared to free drug combinations, DMPLN formulations synchronized the PK of DOX and MMC, ratio-metrically delivered higher concentrations of DOX and MMC into the tumor with sustained local bioavailability of the drugs, and minimized the formation of DOXOL in the heart tissue. As a result, higher tumor cell apoptosis and non-detectable systematic and organ toxicity were observed as compared to the free drug cocktail. Superior pharmacokinetics and biodistribution and efficacy of DMPLN pave the way to the improved design of future nanomedicine in which the drug combination mechanisms, coordinated drug release from nanoparticles, and assurance of intracellular drug delivery into cancer cells should be considered.

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Figure Legends

Figure 1. A) Particle properties of DMPLN and ICG-DMPLN; B) Particle size distribution of DMPLN determined by Zetasizer; C) Kinetics of drug release from DMPLN or free Dox-MMC into pH 7.4 PBS determined using a dialysis tube at 37 °C. Data are mean $\pm$ SEM (n=3).

Figure 2. Real time *in vivo* whole body fluorescence imaging of ICG-DMPLN and *ex vivo* fluorescence imaging of free ICG and ICG-DMPLN in EMT6 breast tumor bearing *Balb/c* mice. A) Whole animal biodistribution of ICG-DMPLN from 0.25-24 h post *i.v.* injection with the baseline being determined prior to ICG-DMPLN injection. Breast tumor is indicated with a red dashed line circle at baseline. B) *Ex vivo* biodistribution of free ICG and C) ICG-DMPLN in major organs and breast tumor 0.25 h after injection. All organs (heart, lungs, spleen, kidneys, liver and intestine) and breast tumor were perfused with ice-cold saline before being resected from the treated mice. Fluorescence intensity was expressed as radiant efficiency (photons/sec/cm²/sr)/ (μ W/cm²).

Figure 3. *Ex vivo* biodistribution of ICG-DMPLN in A) blood, B) breast tumor, and C-H) major organs over 24 h. A single *i.v. injection* of ICG-DMPLN was given to the mice. Animals were sacrificed at predetermined time points over 24 h. Whole blood was withdrawn, and the organs were removed after perfusion with ice-cold saline. The organs and blood were imaged immediately. The relative fluorescence intensity was calculated by comparing to untreated whole blood per ml and normal organs per gram tissue as a function of time. The enlarged representations for heart and spleen were shown in the inset. Data are means $\pm$ SEM (n=3).

Figure 4. A) DOX and MMC concentrations (diamonds for free DOX-MMC and squares for DMPLN) in whole blood as a function of time as determined by LC-MS/MS; B) Total DOX

concentrations; C) Total MMC concentrations; D) Comparison of total DOX and MMC tumor accumulation between the free DOX-MMC combination and DMPLN; E) The molar ratio of DOX and MMC delivered into tumors as free or nanoparticle formulations. All mice bearing EMT6 breast tumor was *i.v.* injected at dosages of 9.2 mg/kg DOX with 2.9 mg/kg MMC for free DOX, PLD (Caelyx[®]), free DOX-MMC, or DMPLN. The tumor accumulation of DOX and MMC was measured at 0.5, 6 and 24 h post *i.v.* injection. For nanoparticle formulations of DMPLN and PLD (Caelyx[®]), the drugs measured include both released and non-released forms. All data are means $\pm$ SEM (n=3).

Figure 5. *In vivo* formation of the DOX metabolite, DOXOL, in EMT6 breast tumors at 0.5, 6, or 24 h after a single *i.v.* injection of free DOX, free DOX-MMC or either of the nanoparticle formulations, PLD (Caelyx[®]) or DMPLN, containing 9.2 mg/kg DOX or 9.2 mg/kg DOX plus 2.9 mg/kg MMC, respectively. A) Total DOXOL concentrations; B) The percentage of DOXOL formed from DOX delivered by PLD (Caelyx[®]) (triangles) or DMPLN (squares). Data are presented as means $\pm$ SEM (n=3).

Figure 6. Caspase-3 activation in EMT6 breast tumors 24 and 48 h post-injection of various DOX or DOX-MMC combination formulations at the dosage of 9.2 mg/kg DOX or plus 2.9 mg/kg MMC. A) Immunohistochemical staining (brown color) for caspase-3 in breast tumor sections (10 $\times$ magnification); B) Percent of positive caspase-3 staining area in the tumor area within the region of interest (ROI) in breast tumor 24 and 48 h post-injection (Supplementary Materials -Figure 6S). * indicates statistically significant difference between DMPLN group and the other groups with $p < 0.05$ at 24 h post-injection; ** indicates statistically significant

difference between DMPLN group and the groups treated by saline and free DOX-MMC combination with $p < 0.05$ at 48 h post-injection.

Figure 7. DOX and DOXOL concentrations in heart of EMT6 breast tumor bearing *Balb/c* mice.

A) DOX and B) DOXOL concentrations in heart at 0.5, 6 or 24 h after a single *i.v.* injection of free DOX, PLD (Caelyx[®]), free DOX-MMC or DMPLN at the DOX dosage of 9.2 mg/kg and MMC dosage of 2.9 mg/kg MMC for free DOX-MMC and DMPLN. The data are means $\pm$ SEM (n=3).

Figure 8. Toxicity evaluation of single DOX or DOX-MMC combination formulations. A).

Plasma cardiac troponin I (cTnI) concentrations were significantly different between saline and treated groups with $p < 0.05$ and $** p < 0.01$; B). Percent of body weight change post-treatment over a 5 day period. All breast tumor bearing mice treated with a single *i.v.* dose of DOX (17.2 mg/kg) or in combination with MMC (5.8 mg/kg) in various formulations were sacrificed on day 5 post-injection. Data were expressed as mean $\pm$ SEM (n=3); C). Sections of heart, liver and kidneys at 40 $\times$ magnification. In the heart, myocardiocyte vacuolation is indicated by black arrows. In the liver, increased centrilobular hepatocyte binucleation and cytoplasmic basophilia are evident in the free Dox and free Dox-MMC groups (arrows); Note also the congested central veins (CV). Minimal periportal hepatocellular hypertrophy is seen in the PLD group (arrows). In the kidney, intraluminal protein casts are evident in free Dox and free Dox-MMC group (arrows).

Table 1. Fitted pharmacokinetic parameters of doxorubicin and mitomycin C in whole blood of breast tumor bearing mice after a single intravenous administration of different single DOX and DOX–MMC combination formulations at the dosage of 9.2 mg/kg DOX and 2.9 mg/kg MMC

ANTICANCER DRUGS [*]	DOXORUBICIN (DOX)				MITOMYCIN C (MMC)	
Formulations	Free DOX	PLD (Caelyx [®])	Free DOX-MMC	DMPLN	Free DOX-MMC	DMPLN
CL (mL•h ⁻¹) [†]	66.8	0.25	67.5	23.2	306.8	43.1
AUC _{0-∞} (μg•h•mL ⁻¹) [‡]	2.7	711.6	2.7	7.7	0.19	1.3
V ₁ (mL) [§]	79.1	0.65	165.1	59.4	491.3	48.1
V _{ss} (mL) ^{**}	419.4	12.7	2167.3	652.9	NA	956.3
t _{1/2α} (h) ^{††}	0.10	0.06	0.40	0.27	NA	0.46
t _{1/2β} (h) ^{‡‡}	5.6	32.9	31.6	24.4	1.1	35.9

^{*}The mean data (n=3) for each time point was used to fit with ADAPTS[®]. DOX used the two-compartment model and MMC in free DOX-MMC formulation used the one-compartment model.

[†]CL is the total body clearance and determined according to CL = dose / AUC_{0-∞}.

[‡]AUC_{0-∞} is area under the curve from time 0 to infinity for the total drugs and was calculated from AUC_{0-∞}=A/α + B/β where A and B are coefficients and α and β are hybrid constants.

[§]V₁ is defined as volume of the central compartment.

^{**}V_{ss} is the volume of distribution at steady state and calculated according to V_{ss} = V₁ × (1 + k₁₂/k₂₁) where V₁ is the volume of central compartment and k₁₂ and k₂₁ are micro-constant (Table 3S –Supplementary Material).

^{††}t_{1/2α} represents the distribution of half-life. In the one-compartmental model, t_{1/2α} is nonexistent.

^{‡‡}t_{1/2β} represents the elimination half-life.

Table 2. Histopathological findings and severity scores in the heart, liver, kidney in the various DOX-based formulations

Treatment [*]	Heart	Liver	Kidney	
	Cardiomyocyte vacuolation [†]	Single cell necrosis [‡]	Glomerular hyaline droplets [§]	Intraluminal protein casts ^{††}
Saline	ND	ND	ND	ND
Free DOX	3 (3;4)	1 (1;1)	1 (1;1)	3.5 (3;4)
PLD (Caelyx [®])	2.5 (2;3)	ND	1 (1;1)	1 (1;1)
Free DOX-MMC	2.5 (2;3)	1 (1;1)	0 (0;0)	3 (2;4)
DMPLN	1 (1;1)	ND	ND	ND

* Median (minimum;maximum) from n=3-4 specimens; Severity score was defined as 0= no lesions; 1= minimum; 2=mild; 3=moderate; 4=marked; ND indicates non-detectable.

[†] Cardiomyocyte vacuolation (% affected cardiomyocytes): 1 = 1-5%; 2 = 5-10%; 3 = 10-25%; 4 = greater than 25%.

[‡] Liver single cell necrosis: 1= Dead hepatocytes were rarely observed (1 per 10 high power field/400x).

[§] Glomerular hyaline droplets: 1= detectable in occasional glomerulus.

^{††} Intraluminal protein casts: 1 = detected in less than 5% of the tubular profiles; 2 = detected in 5-25% of the tubular profiles; 3 = detected in 25-50% of the tubular profiles; 4 = detected in greater than 50% of the tubular profiles.

Figure 1

A

PLN Formulation *	Size (nm)	Zeta Potential (mV)	Polydispersity Index	Drug Encapsulation Efficiency % [†]		
				DOX	MMC	ICG
DMPLN	146 (20)	-22.7 (0.9)	0.37 (0.05)	92.0 (0.9)	50.6 (1.3)	NA
ICG-DMPLN	154 (6.4)	-25.2 (2.6)	0.36 (0.04)	94.1 (2.0)	43.1 (1.9)	88.4 (4.0)

* Data is the mean (standard error of the mean, SEM) with n=3

[†] Drug encapsulation efficiency % = (Drug encapsulated into PLN / Total drug added) × 100

B

C

Figure 2

Figure 3

Figure 4

E

The molar ratio of doxorubicin (DOX) and mitomycin C (MMC) in breast tumor over 24 h

Time (h)	DOX: MMC Molar Ratio [*]	
	Free DOX-MMC	DMPLN
0.5	0.33 $\pm$ 0.19	1.24 $\pm$ 0.17
6	0.43 $\pm$ 0.21	3.43 $\pm$ 0.38
24	N/A [†]	3.01 $\pm$ 0.38

^{*}The molar ratio was calculated based the amount of DOX and MMC per gram of breast tumor. The data represents mean $\pm$ SEM (n=3).

[†] At 24 h, MMC concentration was zero, hence DOX/MMC ratio was not available; otherwise it would be infinite

Figure 5

Figure 6

A

B

Figure 7

A

B

Figure 8