Translational Studies of Phenotypic Probes for the Mononuclear Phagocyte System and Liposomal Pharmacology


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Phenotypic Probes of Liposomal Pharmacology

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List of Abbreviations:
MO/DC = monocytes and dendritic cells
PEG = polyethylene glycol
MPS = mononuclear phagocyte system
PPE = palmar-plantar erythrodysesthesia
ANC = absolute neutrophil count
EOC = epithelial ovarian cancer
PMNs = polymorphonuclear leukocytes (PMNs)
ROS = reactive oxygen species (ROS)
PFS = progression free survival
PLD = PEGylated liposomal doxorubicin
SSC = side scatter
FSC = forward scatter
FITC = fluorescein isothiocyanate
FCM = flow cytometry
AUC = area under the concentration versus time profile
ABSTRACT

Purpose: As nanoparticles (NP) are cleared via phagocytes of the mononuclear phagocyte system (MPS), we hypothesized that the function of circulating monocytes and dendritic cells (MO/DC) in blood can predict NP clearance (CL). Methods: MO/DC phagocytosis and reactive oxygen species (ROS) production were measured in mice, rats, dogs, and patients with refractory solid tumors. PK studies of PEGylated liposomal doxorubicin (PLD), CKD-602 (S-CKD602), and cisplatin (SPI-077) were performed at the MTD. MO/DC function was also evaluated in patients with recurrent epithelial ovarian cancer (EOC) administered PLD. Results: Across species, a positive association was observed between cell function and CL of PEGylated liposomes. In patients with EOC, associations were observed between PLD CL and phagocytosis \( R^2 = 0.43, P= 0.04 \) and ROS production \( R^2 = 0.61, P= 0.008 \) in blood MO/DC. Conclusions: These findings suggest that probes of MPS function may help predict PEGylated liposome CL across species and PLD CL in patients with EOC.
INTRODUCTION

Nanoparticles (NPs), which include PEGylated liposomes, are novel drug delivery platforms that have the potential to improve tumor drug exposure and reduce accumulation in normal tissues more so than their small molecule counterparts (Zamboni and Tonda, 2000; Zamboni, 2005; Zamboni, 2008). The pharmacokinetics (PK) of NPs is dependent upon the carrier and not the drug encapsulated within the carrier until the drug gets released from the carrier (Papahadjopoulos et al., 1991; Park et al., 2004; Zamboni, 2005; Zamboni, 2008). The drug that remains encapsulated within NPs is an inactive prodrug, and thus the drug must be released from the carrier in order to be active. After the drug is released from the carrier, the PK disposition of the drug will be the same as that following administration of the non-carrier form of the drug (Zamboni and Tonda, 2000; Zamboni, 2005; Zamboni, 2008).

The PK disposition of PEGylated liposomal formulations of CKD602 (S-CKD602), doxorubicin (Doxil®), and cisplatin (SPI-077), have been evaluated in preclinical models and patients (Anonymous, 2003; Lee et al., 2000; Zamboni et al., 2007). The ability to extrapolate animal data to predict PK parameters in humans is an essential step in drug development (Gabizon et al., 2008). We have previously explored the use of allometric scaling to predict the PK of PEGylated liposomal agents across species (Caron et al., 2011). Our study indicated that while a relationship exists between species body weight and clearance (CL), there is considerable variability in PK among species, particularly when scaled by conventional and non-conventional parameters. Thus, the development of new methods of scaling and/or measures of NP interaction at the biological level are warranted to further explore the variability observed in NP PK.

Studies suggest that the significantly high and clinically relevant interpatient variability in the PK and pharmacodynamic (PD) disposition of NP anticancer agents is related to the function of monocytes and dendritic cells (MO/DC) of the mononuclear phagocyte system (MPS) (Zamboni et al., 2011a; Zamboni et al., 2011b). The clearance of PEGylated and (No Reference Selected)(No Reference Selected)non-PEGylated nanoparticle agents are cleared via the MPS but PEGylated nanoparticle agents are cleared at a slower rate because of delayed and/or reduced recognition by the MPS (Caron et al., 2012; Dobrovolskaia et al., 2008). The MPS is defined as a group of cells having the ability to ingest large numbers of particles (Hume et al.,
2002). These cells, comprised of MO/DC circulating in the blood, fixed macrophages of various connective tissues, Kupffer cells in the liver, and macrophages in the lymph nodes, bone marrow, and spleen, serve as a potential CL pathway for NPs (Dobrovolskaia et al., 2008; Hume et al., 2002; Lichanska et al., 1999). We have previously reported a significant relationship between the PK and PD of S-CKD602 and changes in circulating monocyte numbers and absolute neutrophil count (ANC) (Zamboni et al., 2011b). The results of our previous study suggest that monocytes are more sensitive to toxic effects of S-CKD602 compared with neutrophils and that the increased sensitivity appears to be related to the liposomal formulation and not the small molecule drug, CKD-602, encapsulated inside the liposome. Thus, blood monocytes may play a key role or be a surrogate marker for NP CL in patients.

Epithelial ovarian cancer (EOC) is a disease characterized by large numbers of peritoneal MO and macrophages, the primary cells of the MPS (Bookman, 2005). As a result of high relapse rates several chemotherapeutic strategies have been developed for this patient population. PEGylated liposomal doxorubicin (PLD) is frequently used second- and third-line for the treatment of recurrent EOC and is one of the few FDA approved NPs currently available (Caron et al., 2012). However, in the second-line treatment of platinum refractory EOC, PLD achieves overall response rates of only 14-20% (Bookman, 2005; Ozols et al., 1997). Moreover, there is significant variability in the PK and PD associated with PLD. Therefore, some patients are much more likely to receive a non-efficacious or toxic, particularly palmar-plantar erythrodysethesia (PPE), response (La-Beck et al., 2011). Thus, there is a compelling need to guide PLD dosing in order to improve the response rate and quality of life for women with EOC (Anonymous, ; Amantea et al., 1997; Gabizon et al., 1994; Gabizon et al., 2008; Uziely et al., 1995).

One approach to improve the treatment of patients is to identify and employ a phenotypic probe to individualize therapy (van der Bol et al., 2010). A phenotypic probe is a test or agent than can be administered to a patient as an indicator of the PK and/or PD of a drug, which can then be used to individualize therapy. Phenotypic probes measuring cellular function in blood could be used to evaluate the relationship between activity of the MPS and the effect on NP PK and PD in a relatively noninvasive fashion. The objective of this study was to evaluate phenotypic probes of MPS function in blood as predictors of PK and PD of PEGylated
liposomal agents in animal models and patients. The function of MO/DC and polymorphonuclear leukocytes (PMNs) of the MPS in blood was evaluated using phagocytosis and reactive oxygen species (ROS) production.

**METHODS**

The preclinical studies were approved by the UNC Institutional Animal Care and Use Committee (IACUC). The clinical studies were approved by the Committee for the Rights of Human Subjects (Institutional Review Board) at the University of North Carolina at Chapel Hill. All patients were advised of the purpose, procedures and associated risks and gave written informed consent.

**Phenotypic and PK Studies in Preclinical Animal Models and Patients**

Phenotypic studies of MPS function and PK of PEGylated liposomal agents were performed in mice, rats, dogs and patients with refractory solid tumors as part of clinical phase I studies (Gabizon et al., 2003; Gabizon et al., 2008; Meerum Terwogt et al., 2000; Meerum Terwogt et al., 2002; Zamboni et al., 2007). A blood sample was obtained using a sodium heparinized tube from each of the species in order to assess MO/DC phagocytosis and ROS production using FCM (methods are detailed in EOC clinical study). Blood samples were obtained prior to administration of liposomal agents in triplicate in each species.

S-CKD602, PLD, and SPI-077 were administered to SCID mice, Sprague-Dawley rats, beagle dogs and as part of phase I clinical studies as described previously (Gabizon et al., 2003; Gabizon et al., 2008; Lee et al., 2000; Meerum Terwogt et al., 2000; Meerum Terwogt et al., 2002; Zamboni et al., 2007). Serial blood sampling times and analytical methods used to determine sum total nanoparticle concentrations are also provided in these previously published studies. The concentration versus time data was imported into Phoenix WinNonlin Version 6.1 (Pharsight Corp., Mountain View, CA) and a noncompartmental analysis was used to determine CL in each species.

**PLD PK and PD Studies in EOC**

**Inclusion Criteria**
Women receiving PLD as part of their standard of care treatment for recurrent EOC were eligible for enrollment in this study. Doxil®, doxorubicin encapsulated in STEALTH® liposomes was purchased from Janssen and used in all patients. Patients had to be ≥ 18 years of age and have a documented hysterectomy or negative pregnancy test.

Clinical Study Design

Baseline characteristics and treatment regimens of the 10 women enrolled are listed in Table 2. Patients were administered standard pre-medications including dexamethasone 10 mg, diphenhydramine 25 mg, famotidine 20 mg, and ondansetron 8 mg all IV x 1 30 minutes prior to PLD. Patients were administered PLD at 40 mg/m² alone or PLD at 30 mg/m² IV x 1 over approximately 1 hour in combination with carboplatin infused IV x 1 over 30 minutes at a dose to achieve AUC = 5 (Calvert equation). Serial blood PK samples were obtained at baseline prior to the administration of PLD or PLD with carboplatin, at the end of infusion (EOI), and 1, 3, 24, 48, 72, 96, 192, and 672 hours after the administration of PLD. Plasma was processed immediately and the encapsulated and released components of PLD were separated using solid phase separation methods as described previously (Zamboni and Tonda, 2000; Zamboni et al., 2007; Zamboni et al., 2009). Noncompartmental analysis was performed using Phoenix WinNonlin Version 6.1 to calculate PK parameters (Table 3).

Blood (3 mL) was obtained at baseline, 48, 72, and 96 hours to test the function of MO/DC. At each visit, vital signs were obtained, physical examinations and blood work was performed at the discretion of the individual physician, and patients were asked about any adverse symptoms they experienced, including but not limited to nausea/vomiting, PPE, neuropathy, and stomatitis. Grade of toxicity was determined by the National Cancer Institute’s Common Terminology Criteria for Adverse Events (NCI CTCAE, version 4.03). Patients were followed until disease progression or toxicity necessitated discontinuation of PLD. Progression free survival (PFS) was determined by RECIST criteria (version 1.1).

Phenotypic Probes
Innate immune function (phagocytosis and ROS production) of peripheral blood monocytes and PMNs was assessed by flow cytometry. Initially data were used to determine whether there was a relationship between cellular function and PK among species. A single 1 mL blood sample was taken from each species (at n= 3) used in the PK studies to test cellular function. Data were then used to assess possible correlations between cellular function (phagocytosis and ROS production) and PK (CL of PLD) and PD (PFS and PPE) of PLD in women with recurrent EOC. Studies of MPS function prior to the administration of PLD in each patient were used to predict PLD PK and PD. Additionally, the changes in cellular phagocytosis and ROS over time was assessed within and between all patients.

Flow cytometry was performed in the UNC Flow Cytometry Core Facility using a Dako Cyan flow cytometer and data were analyzed using FlowJo software v 7.6.5. For both the phagocytosis and ROS assays, MO/DC and PMN populations were gated based on light scatter properties (FSC vs. SSC) and subsequently plotted for histogram analysis (Supplemental Figure 1). The proportion of positive cells (i.e. cells which exhibit fluorescence) was determined as those events, which shifted to the right out of the “negative” region on the fluorescence intensity scale (FITC). Mean fluorescent intensity (MFI) of the positive cell population served as an index of phagocytic or ROS activity.

**Phagocytosis Assay**

Twenty µL of FITC-labeled opsonized *E. Coli* bacteria bio-particles (1 x 10^8 particles/mL) (Orpegen Pharma, San Diego, CA) were added to 100 µL of whole blood and incubated for 10 minutes at 37°C. Additional samples kept on ice (0°C) served as a negative control. After incubation, 100 µL of Trypan blue was added to quench extracellular fluorescence. Phagocytic activity (number of bacteria internalized per cell) was quantified as the MFI of the “positive” cells.

**ROS Production Assay**

ROS was assessed in MO/DC in response to no stimuli and to a variety of stimulants, including opsonized non-fluorescent *E. Coli* as a phagocytic stimulus, N-formyl-methionine-leucine-phenylalanine (fMLP)
as a physiologic peptide, phorbol myristate acetate (PMA) a synthetic ester, and PBS as a control (no stimulus; baseline measurement). Following a 10 minute exposure to the stimulus, non-fluorescent dihydrorhodamine (DHR) 123 (Orpegen Pharma, San Diego, CA) was added to the samples as a fluorogenic substrate, which, following intracellular oxidation was converted to fluorescent rhodamine (R) 123. MFI of R 123 fluorescence served as a quantitative measure of intracellular oxidative activity.

Statistics

All statistical analyses were performed using SAS v 9.2 (Cary, NC) software. Simple linear regression was used to explore the linear relationship between two continuous variables, including the relationship between MO/DC or PMN cellular function and PK (CL) or PD (PPE grade and PFS) The coefficient of determination, R-squared ($R^2$) was used to measure the linear association between PK/PD outcomes and cellular function. The relationship between CL and phagocytosis was evaluated using multiple linear regression including a term for treatment type (PLD vs. PLD + carboplatin). A Kruskal-Wallis test was used to test for differences in median MFI between patients and within patients over the course of cycle 1. A Cox proportional hazards model, using progression-free survival as the outcome variable and phagocytosis as a covariate, was used to estimate predicted progression-free survival at differing levels of phagocytosis. Alpha was set at 0.05 for all statistical tests and all p-values are two-sided.

RESULTS

Relationship Between Cellular Function and PEGylated Liposome PK in Preclinical Models and Patients with Refractory Solid Tumors

The relationship between phenotypic probes of MPS function and PK of PEGylated liposomal agents was evaluated in preclinical tumor models and in patients with refractory solid tumors as part of phase I studies of PEGylated liposomal doxorubicin (Doxil®, PLD), CKD-602 (S-CKD602), and cisplatin (SPI-077). There was a direct linear relationship between MPS activity and the CL of PEGylated liposomes across mice, rats, dogs and
humans. The average Mean Fluorescence Intensity (MFI) in the MO/DC population following the phagocytosis assay in the 4 species evaluated was correlated with CL of PLD ($R^2 = 0.95$), S-CKD602 ($R^2 = 0.99$), and SPI-077 ($R^2 = 0.73$) as shown in Figure 1A. There was a similar trend observed when comparing the production of ROS across species without any stimulus (baseline) with CL of PLD ($R^2 = 0.77$), S-CKD602 ($R^2 = 0.77$), and SPI-077 ($R^2 = 0.66$) (Figure 1B). The relationship was also seen between production of ROS when stimulated with PMA and CL of PLD ($R^2 = 0.83$), S-CKD602 ($R^2 = 0.84$), and SPI-077 ($R^2 = 0.69$).

**Phenotypic Probes Predict PLD PK in Patients with EOC**

The relationship between phenotypic probes of MPS function and PLD PK was evaluated in patients with EOC. On day 1 of the study, phagocytosis and ROS production were assessed in MO/DC prior to the start of the PLD infusion in patients with EOC ($n=10$). A linear relationship ($R^2 = 0.43$, $P= 0.04$) was found between MFI of the phagocytic cells and PLD CL for all patients, shown in Figure 2A. A relationship between MFI of ROS production without stimulus at baseline and PLD CL for all patients was also observed as shown in Figure 2B ($R^2 = 0.61$, $P= 0.008$). The relationship between the MPS function is more significant in patients treated with PLD alone ($R^2 = 0.57$ and $0.61$ for phagocytosis and production of ROS without stimulus, respectively) (Figure 2C and 2D).

There was also a relationship between the ROS probe with the addition of a stimulant, and PLD CL in all patients at baseline (Table 1). The only stimulant that did not show a strong relationship in all patients was PMA, ($R^2 = 0.23$). PMA was also the only probe that had lower association in the PLD alone versus PLD + carboplatin group. However, the other oxidative burst stimulants performed similarly to the phagocytosis probe and also demonstrated stronger relationships in the cohort of patients which received PLD alone vs. PLD + carboplatin.

A multiple linear regression model was also used to examine the relationship between phagocytosis and doxorubicin clearance adjusting for treatment. The model had doxorubicin clearance as the dependent variable and phagocytosis and treatment (an indicator variable for PLD or PLD plus carboplatin) as the independent variables. This model, which results in two intercepts (intercept for PLD alone = $\beta_0$ and intercept
for PLD plus carboplatin = $\beta_0 + \beta_{\text{treatment}}$ and a common slope ($\beta_{\text{phagocytosis}}$), suggests a positive linear association between phagocytosis and CL of PLD ($\beta_{\text{phagocytosis}}=0.04, p=0.07$) where patients with higher MPS function have a higher CL of PLD (Figure 3). Patients on PLD plus carboplatin (dotted regression line) had somewhat lower doxorubicin clearance compared to patients on PLD only (solid regression line), however the treatment effect was not significant ($\beta_{\text{treatment}}=6.06, p=0.38$).

The correlation between either phagocytosis or ROS production in PMNs and PLD CL failed to reach statistical significance in either the total patient population or subpopulations suggesting that PMN are not involved in the PK of PLD. In the study 87.4% ± 10.9% of gated MO/DC in patients tested positive to the phagocytosis or ROS probe. Therefore, differences in MFI between patients were due to cellular function variability and not the ability of the assay to detect positive events.

Cellular Function Over Time in Patients with EOC

The cellular function of MO/DC and PMNs was also assessed over time in the first cycle of PLD with or without carboplatin. Phagocytosis measured in both MO/DC ($P= 0.85$) and PMNs ($P= 0.66$) were not significantly different in patients over the course of measurement (days 1, 3, 5, 28). The same held for ROS (no stimulus) in both MO/DC ($P= 0.37$) and PMNs ($P= 0.25$) over cycle 1. On day 1, just prior to PLD administration, the MFI of ROS (no stimulus) in all patients ranged from 7.4 to 117.05. The mean ± SD MFI of ROS was 39.1 ± 36.4 on day 1. The MFI for ROS in patient 3 was 117.05. Without patient 3, the mean ± SD MFI for ROS of the other 9 patients was 30.5 ± 25.5.

Phenotypic Probes Predict PLD PD in Patients with EOC

All patients enrolled in the study were followed until disease progression and/or PLD related adverse events required discontinuation of PLD treatment. PLD could be stopped for grade 3/4 myelosuppression, stomatitis, PPE, or treating physician discretion. Patient 3 had rapidly progressive disease and died prior to the start of cycle 2 of PLD. Three additional patients (1, 8, and 10) had progressive disease while on PLD. For these four patients, the phenotypic probes of phagocytosis ($R^2= 0.77, P= 0.02$) and ROS ($R^2= 0.67, P< 0.0001$)
prior to PLD administration were predictive of PFS in days (data not shown). A cox proportional hazard model with phagocytosis as the independent variable was fit and we determined the predicted probability of progression-free survival based on the level of MO/DC phagocytosis (Figure 4) using the three quartiles of blood phagocytosis, (Q1=345 (MFI) med=486 (MFI), and Q3= 621 (MFI)).

PLD PK Predicts PD in Patients with EOC

The relationship between PLD PK and PD [Progression Free Survival (PFS) and PPE) was evaluated. There was a significant association observed between encapsulated PLD exposure (AUC) and PFS (days) in the four patients receiving PLD alone who progressed while on PLD treatment \((R^2= 0.88, P< 0.0001)\) (data not shown). For the 5 patients who experienced PPE during the course of the study, there was a non-statistically significant relationship between their exposure to PLD and the highest grade of PPE reported, \((R^2= 0.08, P= 0.6)\) (data not shown)

DISCUSSION

We have previously reported a relationship between physiologic parameters such as body weight, organ blood flow, and monocyte count and the PK of PEGylated liposomes in animal models and in patients with refractory solid tumors (Caron et al., 2011). In this prior study, variability in the PK, particularly CL and exposure as measured by area under the concentration versus time profile (AUC) was noted across species. However, this current study looks at a plausible biological explanation for the variability in PK of NP across species and in a clinically relevant patient population. We found that the phagocytic capacity and level of ROS production in MO/DC in blood of mice, rats, dogs, and humans is correlated with the CL of PEGylated liposomal agents across all species. This finding, in addition to our prior clinical studies of PEGylated liposomal CKD-602 (S-CKD602) prompted the development of a second clinical study that used the same phenotypic probes of MPS function to predict PLD PK and PD in patients with recurrent EOC (Zamboni et al., 2009). For the first time, we have demonstrated that a fast and inexpensive blood test of MPS function obtained prior to the administration of PLD can be used to predict PK, efficacy and toxicity and can be used to individualize
therapy. These probes may also predict PK and PD of other NP, conjugates, monoclonal antibodies and antibody drug conjugates (ADC) in animal models and in patients (Caron et al., 2012).

We observed a linear relationship between MPS activity and the CL of PEGylated liposomes across species. The phagocytic capacity and production of ROS of MO/DC was correlated with CL of the PEGylated liposomes PLD, S-CKD602, and SPI-077. The relationship was particularly noteworthy in MO/DC phagocytosis with the CL of PLD ($R^2 = 0.92$), S-CKD602 ($R^2 = 0.92$), and SPI-077 ($R^2 = 0.77$). This was the first study reporting a relationship between MPS function in blood and CL of a NP across species, including patients with cancer. The phenotypic probes developed in this study can be used to profile various types of NP agents in preclinical models and patients. In addition, the probes of MPS function can be used to determine which animal model(s) predict MPS function and PK and PD of NP in patients.

In order to evaluate the interaction of NPs with the MPS in a clinically relevant patient population and building upon our previous findings, we performed a clinical study using circulating MO/DC in blood as a surrogate measure of the MPS function to predict PLD PK and PD (PFS and PPE toxicity). Results of the study reported here demonstrate that probes of MPS function predict PLD PK and PD. There was a linear relationship between encapsulated doxorubicin CL and both phagocytosis [$R^2 = 0.43$, $P = 0.04$] and ROS activity [$R^2 = 0.61$, $P = 0.008$] in blood MO/DC.

Consistent with the association between MPS probes and PLD PK, there was an association between phagocytosis ($R^2 = 0.77$, $P = 0.02$) and ROS ($R^2 = 0.67$, $P = 0.06$) probes with PFS in the 4 patients who progressed while on PLD alone at the time of manuscript preparation. These results suggest that patients with higher MPS activity have a faster CL of PLD and a lower plasma exposure, which may be associated with less drug being available for delivery to the tumor and lower response. This relationship is further demonstrated by a Cox proportional hazard model that includes all 10 patients and assesses the relationship of the phagocytosis probe and its influence on the outcome of progression-free survival.

Our results suggest that the phenotypic probes may potentially provide valuable information toward dose individualization. Probes could be used to measure MPS function in each patient prior to administration of PLD and then the dose of PLD may be adjusted based on MPS function and target plasma exposure (AUC).
This is a similar process as that used to individualize carboplatin dose based on renal function and target plasma AUC (Calvert et al., 1989; Egorin et al., 1994). One MPS probe also were predictive of PPE toxicity in patients, as the ROS production at baseline was correlated with PPE grade on a scale of 0-5 ($R^2 = 0.56$); however, this will need to be validated in a larger cohort of patients. If probes may be used to determine efficacy, such as PFS, they could also indicate early in the treatment plan whether PLD is a worthwhile option for the particular patient.

When comparing the association between phagocytosis or ROS phenotypic probes and encapsulated doxorubicin CL, patient 3 consistently had the highest value in both measures. All data points were included in this study of 10 patients; however, patient 3 noticeably improves the relationship using simple linear regression. Interestingly, patient 3 had the most extensive disease burden of all patients enrolled. An MRI taken just prior to her start on the study indicated moderate volume ascites and multiple tumor masses abutting the liver, the largest measuring 5.2 x 3.7 cm. She was the only patient enrolled on our study with detectable tumors in her liver. Thus, the higher MPS activity and CL of PLD in this patient may be explained in part by the reported relationship between tumor metastases in liver and the CL of NP (Wu et al., 2011). Our group has previously reported that patients with primary or metastatic tumors in their liver ($n=21$) had a significantly ($P = 0.02$) higher CL of the NP S-CKD602 compared with individuals without tumors in their liver ($n=8$) (22). This suggests that patients with tumors in their liver may require a higher dose of NP compared with patients without tumors in their liver. This is a paradigm shift from what is normally seen with small molecule agents where patients with tumors in their livers have a reduced CL of drugs that are metabolized by phase I and II enzymes (Stewart et al., 1990).

One potential reason for the difference in the relationship between encapsulated doxorubicin CL and cellular functional assays between the PLD only and PLD in combination with carboplatin could be secondary to platinum effects on the cellular function of the MPS cells. The effect of platinum agents on monocytes has been explored in vitro by Nielsen, et al. (Nielsen, 1984). In this study, a 1 µM exposure of cisplatin for 60 minutes was shown to selectively inhibit chemotaxis, which can then also inhibit phagocytosis, in monocytes isolated from venous blood of healthy volunteers (Nielsen, 1984). Fumarulo, et al., also reported an in vitro
chemotaxis inhibition by cisplatin using peritoneal macrophages of the rat (Fumarulo et al., 1980). In addition, an *in vivo* study has reported impaired blood monocyte chemotaxis in cancer patients ≥ 20 hours after receiving cisplatin at 20 mg/m² IV x 1 (Nielsen et al., 1985). Based on these studies and our results, the quick onset of chemotaxis and phagocytosis inhibition by cisplatin could explain a lack of functioning monocytes in the area of drug uptake and subsequently a lower NP CL.

We are aware that, due to the relatively small number of subjects in this study of PLD in patients with EOC, some of our statistical comparisons are likely underpowered, which may have affected our ability to detect significant relationships. Nevertheless, we were able to observe suggestive associations between monocyte function and PLD CL, PFS and PPE toxicity in these exploratory, rather than confirmatory analyses. Moreover, the data show that the patient with the highest probe activity had a different pathophysiology than the other 9 patients enrolled. Not only did this patient have the most extensive disease burden, but the highest ROS measurement. These results may indicate an environment of oxidative stress and imbalanced redox systems (Elbim and Lizard, 2009).

This study shows that the CL of PEGylated liposomes across 4 different species is related to the cellular function of MO/DC. MPS function can be easily measured using flow cytometry (FCM) and serve as a phenotypic probe to relatively non-invasively predict the PK and PD for PLD in women with recurrent EOC. Phenotypic probes are reproducible, highly translatable, and readily transferable to clinical practice as both the cell based assays and flow cytometry analysis are available in hospitals and are straightforward to perform. We demonstrated that phenotypic probes can predict PLD PK, the PLD PK predicts PD and ultimately that the phenotypic probes can predict PLD PD. The ability to employ a clinical test that is fast, inexpensive and can be used to individualize PLD therapy and potentially treatment with other NP agents in patients is of great potential value. A randomized clinical trial comparing response and toxicity of PLD in patients with EOC treated with standard PLD based on body surface area (BSA) compared with the dose of PLD individualized based on our MPS probes is planned.
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Author Contributions:

Participated in research design: Caron, Lay, La-Beck, Clarke-Pearson, Brewster, Van Le, Bae-Jump, Gehrig, Zamboni
Conducted experiments: Caron, Newman, La-Beck
Contributed new reagents or analytic tools: Caron, Lay
Performed data analysis: Caron, Fong, Lay, La-Beck, Kumar, Zhou, Monaco
Wrote or contributed to the writing of the manuscript: Caron, Gehrig, Zamboni
REFERENCES


FOOTNOTES

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b) This work has been previously presented at the American Society of Clinical Oncology (ASCO) Annual Meeting in Chicago, IL in June 2011 and 2012.

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Figure 1. Relationship between phagocytosis (A) and production of ROS (B) in MO/DC from blood and CL of PEGylated liposomal agents in mice, rats, dogs, and patients. The ability to translate results of NP preclinical data to human patients may require measuring cellular function of the cells responsible for NP uptake and CL. The mean value for 3 species are represented by individual symbols, with diamonds as PLD, squares as S-CKD602 and triangles as SPI-077. The species data are in vertical columns from left to right: rats, mice, dogs, and patients. The best fit line for each group is represented by the solid lines. Across species, a positive association was observed between cell function and CL of PEGylated liposomes.

Figure 2. Relationship between MO/DC function and encapsulated doxorubicin CL in patients presented in a linear regression model. Measuring phagocytosis and production of ROS of MO/DC from patient blood samples at baseline (prior to the start of chemotherapy) was used as a phenotypic probe of MPS function and encapsulated doxorubicin CL. Each open circle represents an individual patient and the solid line is the regression line. (A) Phagocytic activity (MFI) is significantly correlated with CL of encapsulated doxorubicin in 10 patients ($R^2 = 0.43$, $P = 0.04$). (B) Production of reactive oxygen species (ROS) (MFI) is significantly correlated with CL of encapsulated doxorubicin in 10 patients ($R^2 = 0.61$, $P = 0.008$). (C) Phagocytic activity (MFI) is significantly correlated with CL of encapsulated doxorubicin in 6 patients receiving PLD alone ($R^2 = 0.57$, $P = 0.03$). (D) Production of reactive oxygen species (ROS) (MFI) is significantly correlated with CL of encapsulated doxorubicin in 6 patients receiving PLD alone ($R^2 = 0.61$, $P = 0.001$).

Figure 3. The multiple linear regression model with doxorubicin clearance as the dependent variable and phagocytosis and treatment (an indicator variable for PLD or PLD plus carboplatin) as independent variables. Individual data points are represented as the symbols. The results suggest a positive linear association between phagocytosis and CL of PLD ($\beta_{\text{phagocytosis}} = 0.04$, $p = 0.07$). The observations for the two treatment types are denoted by x=PLD + carboplatin and open circle = PLD only. The regression lines are displayed for the two treatment types (dotted line = PLD + Carboplatin and solid line = PLD only). This model, which results in two intercepts (intercept for PLD alone = $\beta_0$ and intercept for PLD plus carboplatin = $\beta_0 + \beta_{\text{treatment}}$) and a common slope ($\beta_{\text{phagocytosis}}$), suggests that patients with higher MPS function have a higher CL of PLD. Patients on PLD + carboplatin (dotted regression line) had somewhat lower doxorubicin clearance compared to patients on PLD only (solid regression line), however the treatment effect was not significant ($\beta_{\text{treatment}} = 6.06$, $p = 0.38$).

Figure 4. Progression free survival probability over time in days for 10 patients with recurrent EOC receiving PLD. Using the three quartiles of blood phagocytosis, (Q1=345 (MFI) med=486 (MFI), and Q3= 621 (MFI)), a cox proportional hazard model with phagocytosis as the independent variable can be fit to the data. The model suggests lower progression-free survival probabilities for higher levels of phagocytosis in MO/DC in blood.
Table 1. Ability of an oxidative burst phenotypic probe to predict PLD clearance in patients

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>All Patients (R², rₛ)</th>
<th>PLD Only (R², rₛ)</th>
<th>PLD + carboplatin (R², rₛ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No stimulant (baseline cell ROS production)</td>
<td>0.61, 0.64,‡</td>
<td>0.61, 0.6,*</td>
<td>0.0005, 0.4</td>
</tr>
<tr>
<td>E. Coli (particulate)</td>
<td>0.46, 0.22,*</td>
<td>0.44, 0.2</td>
<td>0.26, 0.4</td>
</tr>
<tr>
<td>fMLP (physiologic)</td>
<td>0.54, 0.57,*</td>
<td>0.48, 0.7</td>
<td>0.14, 0.4,‡</td>
</tr>
<tr>
<td>PMA (synthetic)</td>
<td>0.23, 0.72</td>
<td>0.21, 0.8</td>
<td>0.59, 0.8</td>
</tr>
</tbody>
</table>

‡ P ≤ 0.001
* P ≤ 0.05

Ability of an oxidative burst phenotypic probe to predict PLD clearance in patients. Linear regression of the oxidative burst probes and PLD CL in the 10 patients enrolled in the study. Values are reported for patients who received PLD (only) or PLD + carboplatin as their standard therapy for recurrent EOC.
Table 2. Baseline characteristics of the patients who have been enrolled in the study

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Race</th>
<th>Weight (kg)</th>
<th>BSA</th>
<th>Chemotherapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>63</td>
<td>C</td>
<td>54</td>
<td>1.63</td>
<td>PLD</td>
</tr>
<tr>
<td>2</td>
<td>51</td>
<td>C</td>
<td>73</td>
<td>1.9</td>
<td>PLD</td>
</tr>
<tr>
<td>3</td>
<td>57</td>
<td>C</td>
<td>71</td>
<td>1.78</td>
<td>PLD</td>
</tr>
<tr>
<td>4</td>
<td>67</td>
<td>C</td>
<td>89</td>
<td>1.96</td>
<td>PLD + carboplatin</td>
</tr>
<tr>
<td>5</td>
<td>76</td>
<td>C</td>
<td>91</td>
<td>1.91</td>
<td>PLD</td>
</tr>
<tr>
<td>6</td>
<td>51</td>
<td>C</td>
<td>72</td>
<td>1.84</td>
<td>PLD + carboplatin</td>
</tr>
<tr>
<td>7</td>
<td>53</td>
<td>C</td>
<td>94</td>
<td>1.94</td>
<td>PLD + carboplatin</td>
</tr>
<tr>
<td>8</td>
<td>75</td>
<td>C</td>
<td>48</td>
<td>1.46</td>
<td>PLD</td>
</tr>
<tr>
<td>9</td>
<td>44</td>
<td>C</td>
<td>77</td>
<td>1.91</td>
<td>PLD + carboplatin</td>
</tr>
<tr>
<td>10</td>
<td>52</td>
<td>AA</td>
<td>116</td>
<td>2</td>
<td>PLD</td>
</tr>
<tr>
<td>Mean ± SD Or Total</td>
<td>59 ± 10.9</td>
<td>9 CC 1 AA</td>
<td>78 ± 20</td>
<td>1.8 ± 0.2</td>
<td>6 PLD alone 4 PLD + carboplatin</td>
</tr>
</tbody>
</table>

C = Caucasian, AA = African American
Table 3. PEGylated liposomal doxorubicin Plasma PK Parameters in Patients with Recurrent EOC

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Encapsulated</th>
<th>Released</th>
<th>Ratio of AUC_released to AUC_encapsulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL</td>
<td>(mL/h)</td>
<td>(mL/h)</td>
<td></td>
</tr>
<tr>
<td>AUC</td>
<td>(ng/mL*h)</td>
<td>(ng/mL*h)</td>
<td></td>
</tr>
<tr>
<td>t(_\frac{1}{2})</td>
<td>(h)</td>
<td>(h)</td>
<td></td>
</tr>
<tr>
<td>Vd</td>
<td>(mL)</td>
<td>(mL)</td>
<td></td>
</tr>
<tr>
<td>CL</td>
<td>(mL/h)</td>
<td>(mL/h)</td>
<td></td>
</tr>
<tr>
<td>AUC</td>
<td>(ng/mL*h)</td>
<td>(ng/mL*h)</td>
<td></td>
</tr>
<tr>
<td>t(_\frac{1}{2})</td>
<td>(h)</td>
<td>(h)</td>
<td></td>
</tr>
<tr>
<td>Vd</td>
<td>(mL)</td>
<td>(mL)</td>
<td></td>
</tr>
</tbody>
</table>

Mean ± SD

<table>
<thead>
<tr>
<th>Encapsulated</th>
<th>Released</th>
<th>Ratio of AUC_released to AUC_encapsulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>25.7 ± 12.1</td>
<td>2,898,782 ± 974,518</td>
<td></td>
</tr>
<tr>
<td>79.6 ± 25.3</td>
<td>2,569 ± 502</td>
<td></td>
</tr>
<tr>
<td>253.0 ± 175.8</td>
<td>243,541 ± 171,578</td>
<td></td>
</tr>
<tr>
<td>189.7 ± 252.6</td>
<td>40,166 ± 34,838</td>
<td>0.10 ± 0.05</td>
</tr>
</tbody>
</table>
Figure 1

**A**

- PK (CL mL/h) of PEGylated Liposome vs. Baseline MPS Activity (Phagocytosis MFI)
- Data points for rat, mouse, dog, and patient
- Lines with corresponding R² values: PLD (R² = 0.95), S-CKD602 (R² = 0.99), SPI-077 (R² = 0.73)

**B**

- PK (CL mL/h) of PEGylated Liposome vs. Baseline MPS Activity (ROS Production)
- Data points for rat, mouse, dog, and patient
- Lines with corresponding R² values: PLD (R² = 0.83), S-CKD602 (R² = 0.84), SPI-077 (R² = 0.69)
Figure 2

A) All Patients

Encapsulated doxorubicin Clearance (mL/h) vs. Phagocytosis (MFI)

B) All Patients

Encapsulated doxorubicin Clearance (mL/h) vs. Production of ROS (MFI)

C) PLD Only

Encapsulated doxorubicin Clearance (mL/h) vs. Phagocytosis (MFI)

D) PLD Only

Encapsulated doxorubicin Clearance (mL/h) vs. Production of ROS (MFI)
Figure 3
Figure 4