

Introduction

As pharmaceutical care continues to advance in the area of oncology, scientists have turned to nanoparticle delivery systems to improve upon existing anticancer agents. Exact definitions vary, but one commonly used definition from the National Nanotechnology Initiative defines nanotechnology as “science, engineering, and technology” on the order of 1 to 100 nanometers in any dimension¹. The last two decades have witnessed enormous growth in the development of nanoparticle anticancer agents. In 1995, the FDA approved pegylated liposomal doxorubicin (PLD, Doxil[®]) for the treatment of platinum-refractory ovarian cancer. Since that time, there has been extensive research into the use of liposomal agents for the treatment of various malignancies. The hope has been, and continues to be, that these agents can be used to target cytotoxic chemotherapy to malignant cells while minimizing exposure to healthy cells. Unfortunately, only two other nanoparticle agent, Abraxane[®] and Marqibo[®], have come to market in the 20 years since the FDA approved Doxil[®]. Other agents that have been studied as liposomal formulations but are not currently FDA approved include belotecan, irinotecan, lurtotecan, cisplatin, oxaliplatin, paclitaxel, and vinorelbine².

Nanoparticles, and liposomes in particular, are a favored method of drug delivery because they provide the ability to control the timing and location of a drug's release³. An additional benefit of liposomal agents is reduced clearance compared to small molecule agents, providing more sustained drug concentrations and less frequent dosing⁴. It has been observed for some liposomal agents that concentrations in tumor tissue are 10 to 50 times that observed in circulation⁵. This finding is attributed to the enhanced permeation and retention (EPR) effect. Tumors secrete growth factors like VEGF that promote angiogenesis and help sustain the rapid growth of malignant cells. These new blood vessels are irregular and dilated with wide fenestrations, the epithelial cells are poorly aligned, and many vessels lack an underlying basement membrane or smooth muscle layer⁶. The extravasation of liposomes out of circulation is assisted by an imbalance between vasodilators (e.g. nitric oxide, bradykinin, prostaglandins) and vasoconstrictors (angiotensin II) in tumor tissue. In addition, tumor tissue has slow venous return and poor lymphatic drainage, leading to prolonged retention of liposomes and accumulation of the drug in tumor⁷.

Small molecule drugs are not subject to the EPR effect and are rapidly cleared by the liver or kidney. Liposomes, due to their relatively larger size, are cleared by the immune cells of the mononuclear phagocyte system (MPS). The MPS comprises the circulating blood monocytes as well as resident tissue macrophages⁸. The exact interaction between the MPS and liposomes is still not completely understood, as it involves a complex bidirectional interplay: the MPS cells in the body determine

biodistribution and clearance of the liposome, and the antineoplastic agent encapsulated within the liposome can have a cytotoxic effect on the MPS⁹⁻¹². Because liposomes are cleared from circulation by the MPS, they primarily accumulate in tissues with dense populations of these cells, notably the liver and spleen¹³.

While liposomes and other nanoparticle agents have the advantages discussed above, they have not been more widely developed as commercial agents due to issues of pharmacokinetic (PK) and pharmacodynamics (PD) variability. Multiple studies have compared liposomal agents to their small molecule equivalent and confirmed that liposomes have significantly greater PK and PD variability¹⁴⁻¹⁷. The exact causes are still unclear, but there does appear to be a correlation between PK/PD variability and patient specific factors such as age, body composition, and clearance rate of the drug^{2,17}.

This PK/PD variability may help explain why some cancers respond to liposomal agents while other do not. PLD provides an excellent case study here. The FDA approved Doxil[®] on the basis of a Phase 3 study showing a survival benefit compared to standard of care in platinum refractory ovarian cancer¹⁸. However, these benefits have not extended to other gynecologic cancers. A Phase 2 study designed to evaluate the antitumor activity in patients with persistent or recurrent endometrial carcinoma found limited activity and subsequent studies have not provided sufficient rationale to pursue this research further¹⁹.

The expansion of liposomal anticancer agents to improve on current standard of care would be greatly simplified by a relatively rapid, affordable, and accurate marker to explain PK/PD variability and predict efficacy in specific patient populations. One potential candidate is the F4/80 marker for tumor associated macrophages (TAMs). TAMs are specific MPS cells that migrate to and exert their function within the tumor tissue. They are mediators of inflammation and are associated with poor prognosis²⁰. TAMs regulate tumor growth, angiogenesis, invasion and metastasis through the release of cytokines, chemokines, enzymes, and growth factors²¹. Zamboni et al illustrated that the number of MPS cells associated with different tumor cell types correlated with distribution of a liposomal anticancer agent to tumor tissue, release of the agent within the tumor tissue, and tumor response to the agent²².

The F4/80 marker is highly specific for MPS cells and survives tissue fixation and embedding, allowing the opportunity to visualize and quantify MPS cells in tissue samples⁸. The exact processing and staining methodology has been previously described²³. MPS cells stain at varying levels of intensity, which can be quantified using the H Score calculation of Budwit-Novotny²⁴. Cells are scored as stain: not present (0), weak but detectable (1+), distinct (2+), or strong (3+).

Figure 1: Comparison of a melanoma (A375) and ovarian (SKOV-3) cancer cell line in terms of number of MPS cells, distribution of a liposomal cancer agent in an encapsulated (S-CKD602) and released (CKD-602) form, and tumor response.

(Adapted from: Zamboni, 2011)

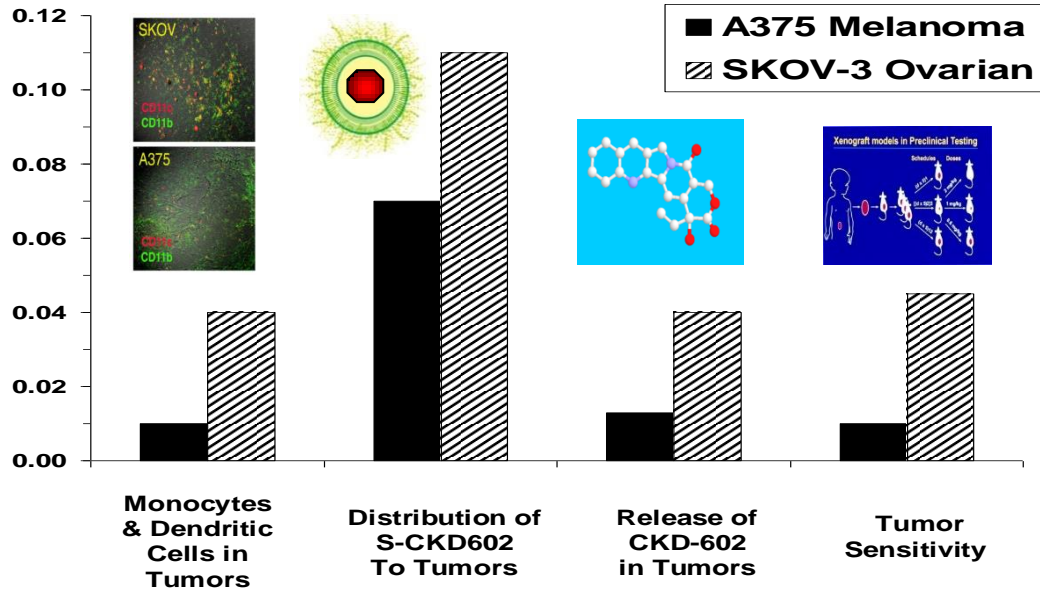


Figure 2: F4/80 staining in tumor tissue, illustrating how the various grades of staining appear before and after computer algorithm analysis.



The following formula is then used to obtain an overall H Score, ranging from 0 to 400, for a tissue region or an entire tissue.

$$H - score = \frac{(\#ofcellsgrade\ 3) \times 4 + (\#ofcellsgrade\ 2) \times 3 + (\#ofcellsgrade\ 1) \times 2}{totalnumberofcells} \times 100$$

The goal of this honors project was to compare the pharmacokinetics and efficacy of PLD to small molecule doxorubicin in two xenograft mouse models, one endometrial (i.e. HEC1A) and one ovarian (i.e. SKOV-3), with differing TAM densities as measured by H Score analysis. PLD concentrations were measured separately for drug still within the liposomal carrier (i.e. encapsulated) and drug released from the liposomal carrier (i.e. released). It is important to note that encapsulated drug is pharmacologically inactive and possesses the PK properties of the carrier molecule, while released drug is active and possesses the PK properties of the small molecule agent¹⁴.

Methods

HEC1A and SKOV3 cells were obtained from the American Type Culture Collection (Rockville, MD) and expanded in culture by the Tissue Culture Facility at the University of North Carolina.

Female CB17 SCID mice aged 8 to 12 weeks were purchased from Taconic Labs (Albany, NY) and were allowed to acclimate for 1 week at the UNC Lineberger Cancer Center prior to initiation of the study. The mice in the endometrial arm were injected with 1,000,000 HEC1A xenograft cells in 25 uL volume into the uterine horn. The mice in the ovarian arm were injected with 200,000 SKOV3 xenograft cells in 5 uL volume into the ovarian bursa.

Animal weights were obtained daily for 2 weeks, then biweekly for the remainder of the study. Tumor dimensions were measured by palpation biweekly for the duration of the study. Animals were sacrificed if tumors measured 2.0 cm in any direction or the animal lost 20% from maximum body weight achieved.

PLD was purchased from FormuMax Scientific (Palo Alto, CA) and nonliposomal doxorubicin (NL-doxo) was purchased from Sigma Aldrich (St. Louis, MO). Dosing details for each phase of the study are provided in **Table 1**. In the PK portion of the study, drug was injected IV x 1 via a tail vein. In the efficacy portion of the study, drug was injected IV via a tail vein weekly for up to 6 doses or until the animal met criteria for harvest.

Table 1: Dosing of PLD and NL-doxorubicin by study phase

Phase	PLD diluted in	PLD diluted to	NL-doxo diluted in	NL-doxo diluted to
PK	D5W	1.2 mg/mL	NS	1.2 mg/mL
Efficacy 6 mg/kg	D5W	1.2 mg/mL	NS	1.2 mg/mL
Efficacy 3 mg/kg	D5W	0.6 mg/mL	NS	0.6 mg/mL

In the PK and initial efficacy studies, mice were treated with PLD and NL-doxorubicin at 6 mg/kg. Mice in the initial efficacy study did not tolerate multiple doses, so a follow up efficacy study was conducted comparing PLD and NL-doxorubicin at 3 mg/kg. In addition to the PLD and NL-doxorubicin arms for both mice bearing HEC1A and SKOV3 xenografts, the efficacy studies included a “control” arm consisting of tumor bearing mice who received no treatment.

PK samples were obtained prior to drug injection and at 5 min, 30 min, 1 hr, 3 hr, 6 hr, 24 hr, 48 hr, 72 hr, and 96 hr post injection. Blood samples were placed on ice and processed the same day using a solid phase separation method described previously to isolate encapsulated and released drug fractions²⁵. Tissues were snap frozen after collection and stored at -80°C until further processing. They were then homogenized with pH 7.4 PBS buffer at a 1:3 ratio of tissue:PBS and drug in tissue was isolated and measured using protein precipitation. Released doxorubicin in plasma, encapsulated PLD in plasma, and sum total doxorubicin in tissues were all measured using a high performance liquid chromatography assay²⁵. Areas under the curve (AUCs) for overall drug exposure were calculated using Phoenix Version 6.2 (Pharsight Corp) with a linear up, log down rule.

Results

PLD concentrations as measured in plasma, liver, and spleen were not significantly different between the mice bearing HEC1A and SKOV3 xenografts. AUC analysis showed that SKOV3 PLD exposures were 70%, 86%, 102%, and 143% of the HEC1A exposures for released drug in plasma, encapsulated drug in plasma, sum total drug in liver, and sum total drug in spleen, respectively. This compared to an SKOV3 PLD AUC in tumor tissue that was 263% of HEC1A AUC. PK data for plasma and tumor are plotted with respect to time in **Figure 3** and **Figure 4**. At 3 hours, concentrations in SKOV3 tumor tissue averaged 11.2 times that in HEC1A tumor tissue. This differential gradually narrowed over time, but was still observed as far out as the 48 hour time point.

Figure 3: Concentration vs. Time in plasma from 0 to 96 hours after administration of PLD at 6 mg/kg x 1 dose in female SCID mice bearing SKOV3 and HEC1A xenografts. Enc: Encapsulated PLD fraction. Rel: Released PLD fraction

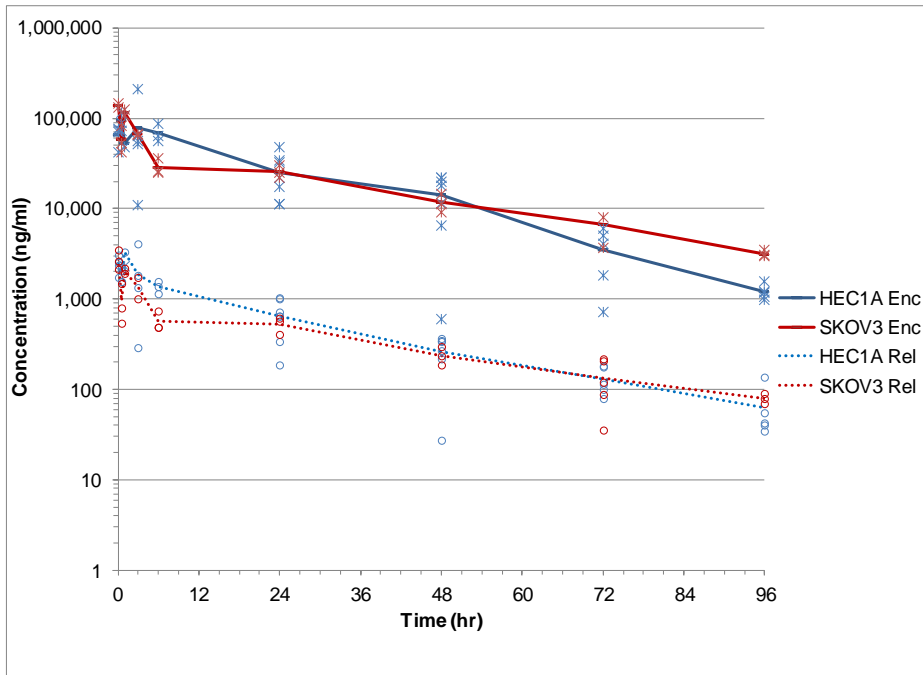


Figure 4: Concentration vs. Time in tumor from 0 to 96 hours after administration of PLD at 6 mg/kg x 1 dose in female SCID mice bearing SKOV3 and HEC1A xenografts. Tot: Sum total doxorubicin

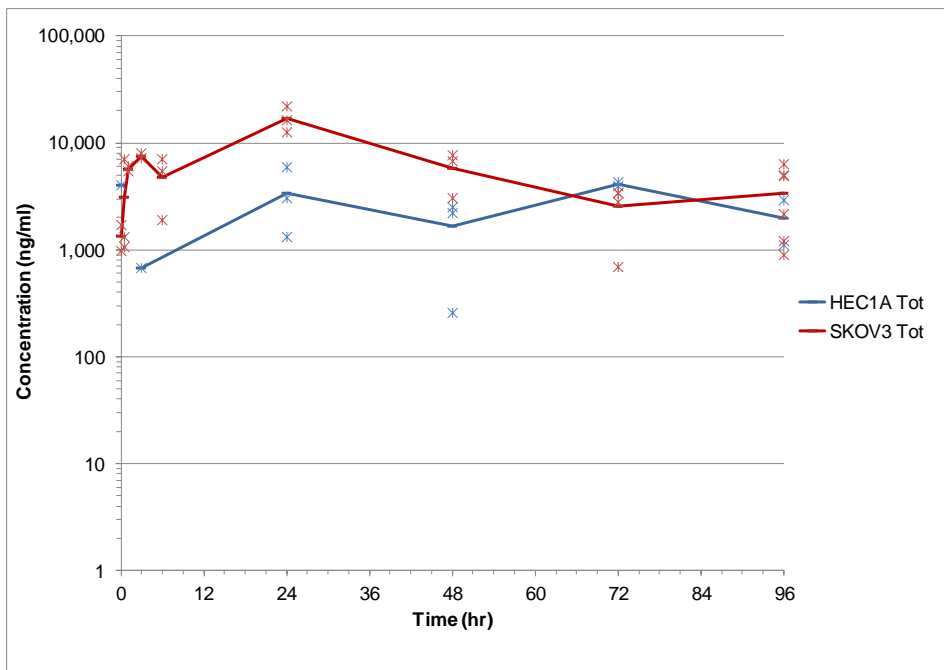
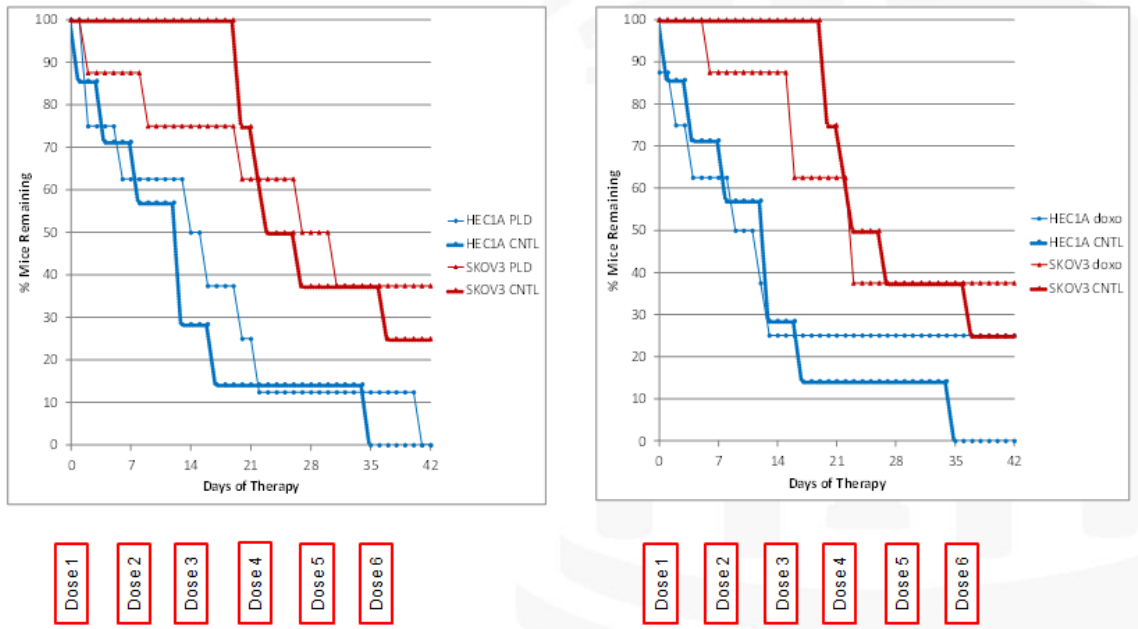


Figure 6: Survival data in mice carrying either SKOV3 ovarian or HEC1A endometrial xenografts who received pegylated liposomal doxorubicin (PLD) at 3 mg/kg, nonliposomal doxorubicin (doxo) at 3 mg/kg, or no treatment.



Accordingly, a follow up efficacy study was conducted at 3 mg/kg. These results are presented in **Figure 6**. In this study, median survival for HEC1A PLD vs. NL-doxorubicin vs. control was 14 days, 13 days and 13 days. Median survival for SKOV3 PLD vs. NL-doxorubicin vs. control was 27 days, 23 days and 20 days. With the limited number of mice in each group, the potential trend toward improved survival in the SKOV3 mice treated with PLD did not reach statistical significance ($p = 0.85$).

Discussion

Our findings in this study present more than one paradox. The pharmacokinetic data suggests a link between MPS function, as measured by the H score, and drug distribution to xenograft tumor tissue. Mice carrying representative ovarian (SKOV3) and endometrial (HEC1A) xenografts were given a single dose of PLD or NL-doxorubicin at 6 mg/kg. Mice tolerated this treatment well up to 96 hours, and data up to this point suggests minimal differences in plasma drug concentrations when comparing the two xenografts either based on released or encapsulated drug. However, the mice with ovarian xenografts, which had more MPS cells as measured by H Score analysis, accumulated more PLD in tumor tissue, suggesting that the increased number of TAMs

migrating to the tumor in SKOV3 mice was helping to deliver the liposomal agent. This same benefit was not seen with NL-doxorubicin.

However, this pharmacokinetic link did not translate to an efficacy advantage at either the initial 6 mg/kg or the subsequent repeat study at 3 mg/kg. Tumor bearing control mice who received no treatment outperformed both treatment groups for both types of xenografts at 6 mg/kg weekly. Moreover, it was noted that the vast majority of mice did not tolerate more than 2 doses of drug, whether liposomal or small molecule, suggesting that acute anthracycline toxicity is playing more of a role here than the cancer progression. The mouse facility noted prior issues with anthracycline toxicity in SCID mice on projects for other researchers; however, this had not previously been observed on Zamboni lab projects as we use other mouse models for these types of studies.

The lower dose of 3 mg/kg also produced disappointing efficacy results. At this dose, SKOV3 mice had improved survival relative to HEC1A mice no matter the treatment arm. Median survival was consistent with the anticipated trend: PLD > NL-doxorubicin > control. However, this trend was not statistically significant. One interesting trend to note when comparing the 6 mg/kg data to the 3 mg/kg data is the fact that survival times for the HEC1A mice remained the same while survival times for the SKOV3 mice improved notably with the dose reduction. One possible explanation is that the survival benefit of PLD is still being obscured to some degree by acute anthracycline toxicity. Less than 1/3 of SKOV3 mice tolerated all 6 doses of PLD, and no HEC1A mice made it this far. The data for NL-doxorubicin is similar.

This study emphasizes several limitations associated with the use of orthotopic xenograft mouse models. While orthotopic models are considered more physiologically accurate than flank models, these models are generally not considered as physiologically accurate as genetically engineered models (GEMMs). Several authors have noted that orthotopic xenografts are not sufficient to fully replicate the complex and heterogeneous tumor environment²⁶⁻²⁸. Additionally, SCID mice are not considered ideal surrogates because they are immunocompromised and have issues with proper DNA repair, resulting in unacceptably high rates of chemotherapy toxicity²⁹. These results are consistent with our own. Nevertheless, xenograft mouse models are considered a rapid, inexpensive, and versatile method to model the development and progression of cancer in humans.

One potential step moving forward is to perform one additional efficacy analysis in mice treated at 1 mg/kg. This dose should be sufficiently low enough that toxicity should not confound the results, allowing any potential survival benefit from PLD to become more clear over the 6 week treatment period.

In summary, this study illustrates a link between the MPS and the distribution of PLD in orthotopic xenograft mouse models. The pharmacokinetic advantage of PLD is associated with tumor cell lines that have higher MPS population and is most likely a function of MPS uptake and delivery of liposomal anticancer agents preferentially to the tumor tissue. Unfortunately, whether this pharmacokinetic advantage translates into a benefit in terms of efficacy is still unknown. Researchers employing SCID mice for efficacy studies should be especially cautious in dosing these animals with anthracyclines, as they are particularly prone to toxicity.

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