Role of Caveolae Mediated Transport for Abraxane Uptake *in vivo*

BY

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THESIS

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This thesis is dedicated to my family, without whom I could not have accomplished this achievement.

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LIST OF ABBREVIATIONS

KO   Caveolin-1$^{-/-}$ (Knockout)

PGG-PTX   Poly-(gamma-L-glutamylglutamine)-paclitaxel

PTX   Paclitaxel

WT   B6/129SF2/J (Wild-Type)

SUMMARY

An *in vivo* study was conducted to determine whether the chemotherapeutic drug Abraxane is taken up by tissues and tumors via caveolae-mediated endocytosis and transcytosis. Cancer is considered as one of the leading causes of death worldwide and is a global health concern due to the increase in global death rates being reported each year. Breast cancer is the most common form of cancer in women worldwide, in both developed and developing counties. Chemotherapeutic drugs are primarily used for treatment of breast cancer. A major focus of basic and clinical research is to find new drug delivery systems that increase the therapeutic efficacy of chemotherapeutic agents while reducing the associated toxicity in normal tissues.

In the present study, we focused on the mechanism of tissue uptake of the chemotherapeutic drug Abraxane, a newly developed drug currently used for treatment for breast cancer. Abraxane is an albumin-bound paclitaxel conjugate specifically designed to replace the commonly used paclitaxel formulation, Taxol, which is delivered in a cremophor-ethanol solution. Taxol requires long infusion cycles and is associated with highly toxic side effects that have been associated with this drug formulation. The proposed internalization of Abraxane into the tissue and tumors, according the pharmaceutical company Celgene, is via a caveolin-1 mediated transport pathway.

Our experimental design included the use of murine models, wild-type B6/129 female mice as compared to female caveolin-1 knockout mice, which were intravenously injected with either paclitaxel suspended in a 1% albumin-saline solution or Abraxane suspended in saline. Blood and tissues were collected and processed for analysis of paclitaxel by liquid

SUMMARY (continued)

chromatography and mass spectrometry. The results obtained showed a higher accumulation of

Abraxane in the liver, kidney, and breast tissues while paclitaxel uptake was greater in the lung

and liver tissues. Plasma levels were higher for Abraxane, suggesting it has a longer circulation

time in the blood. Both Abraxane and paclitaxel accumulated at low levels in the brain

suggesting neither is able to cross the blood-brain barrier. Finally, there were no significant

differences in the accumulation of either paclitaxel formulation between wild type and knockout

mice.

We conclude from this study that the uptake and transport of Abraxane in normal tissues

does is not mediated by caveolae-dependent mechanisms and that the beneficial effects of the

albumin-paclitaxel conjugation of Abraxane may have more to do with its greater bioavailability.

1. INTRODUCTION

1.1 <u>Cancer: (World) Demographics and Epidemiology</u>

Cancer is one of the leading causes of death worldwide (WHO, 2009). The World Health Organization reported that in 2008, 7.6 million or 13% of all deaths worldwide were due to cancer and there is a steady increase in incidence of new cases being reported each year. The World Cancer Report estimated there will be an increase in global cancer incidence of 50% to an astonishing 15 million cases by the year 2020 (Ahmedin et al., 2011; Boyle et al., 2008). One of the main reasons for the increase in cancer rate can be attributed to new cases being reported in developing countries.   Where developed countries had held the majority of the burden previously, the choice of having a "Western-like" lifestyle in developing countries has made its impact in the global burden of cancer (Boyle et al., 2008). Some of the risk factors associated with this type of lifestyle include tobacco and alcohol use, unhealthy diet, and chronic infections. An example where these trends are being observed is breast cancer (Boyle et al., 2008).

Breast cancer is the second most common form of cancer worldwide (Ahmedin et al., 2011; Boyle et al., 2008) and the number one type of cancer in women in both the developed and developing countries (Ahmedin et al., 2011; Boyle et al., 2008). Both incidence and death rate around the world vary widely but the majority of breast cancer cases occur in developing countries (Ahmedin et al., 2011; Boyle et al., 2008). Again, this can be attributed to an increase in life expectancy, urbanization, and embracing a Western lifestyle (Ahmedin et al., 2011; Boyle et al., 2008). Most of these cases are diagnosed in the late stages of the disease where a combination of cost-effectiveness and affordable interventions is lacking. Other risk factors associated directly with breast cancer include hereditary, race and ethnicity, hormones, and

chemicals used in contraceptive medication, hormone therapy after menopause, or diethylstilbestrol (DES) in earlier cases of miscarriage in the 1940-1960s (ACS, 2010a).

In the United States, cancer is the #2 cause of death accounting for 1 out of 4 deaths (ACS, 2010a). There are approximately 1.5 million new cases reported in the United States in 2010. Breast cancer has the highest number of diagnosed new cases and is the second leading cause of death in women. A 2-3% decrease in death rate is attributed to early detection, improved treatment, and lower incidence in recent years (ACS, 2010a).

2. REVIEW OF THE LITERATURE

2.1   Cancer: Treatment – Need for Targeted Drug Delivery

The fight against cancer is a highly studied field all over the world due to its severity and deadly outcome in many cases. While prevention strategies are being put into practice, research has focused on treatment strategies and to find a cure for cancer (Boyle et al., 2008). There is a huge effort to find treatments to contain and eradicate cancer while inducing the least number of side effects and providing a better quality of life. The regime for treating cancer, at the present time, is dependent on the type of cancer and stage (progression) of the cancer in the individual. The general approach for the treatment of cancer is surgery, chemotherapy, and radiation therapy (ACS, 2011). Others less used in treatments include immunotherapy, angiogenesis inhibitors, biological therapy, hyperthermia, bone marrow and peripheral blood stem cell transplantation, photodynamic therapy, hormone replacement therapy, and molecular targeted therapy (ACS, 2011).

Chemotherapy is one of the primary methods used for cancer treatment and recent technology has paved the way for the development of new methods for drug delivery (ACS, 2010b). Chemotherapy is the treatment of cancer using drugs to kill cancer cells by impeding their growth and replication. These drugs are cytotoxic and kill off cancer cells as they circulate through the body in the bloodstream (ACS, 2010b). Chemotherapeutic drugs also kill off healthy cells because they cannot distinguish between a healthy cell and a cancer cell. Cancerous cells can grow much faster than normal cells, and therefore it is though that chemo-drugs kill off more cancer cells than normal cells although they also kills normal cells as well (Persidis, 1999). This can be detrimental to a person's health and cause unpleasant side effects. One way to minimize

these unwanted side effects is by controlling the dosing and frequency of the drug being administered. Although this approach, to reduce side effects, can help increase the comfort level of patients taking chemotherapeutic drugs, it is not necessarily helpful to everyone and some patients continue to suffer. Also, decreasing the dose can minimize the effectiveness of the drug and reduce its ability to fight cancer (Ahmedin et al., 2011; Boyle et al., 2008; ACS, 2010a; WHO, 2011). Another issue is multidrug resistance where cancer develops a resistance to chemotherapeutic drugs. Chemotherapy kills off the drug-sensitive cells but not the drug resistant cells allowing for continuous growth and failed therapy (Persidis, 1999). Given the trade-off between high local concentration in the target tissue and systemically low drug levels, targeted drug delivery becomes a prerequisite for efficient cancer therapy.

Potential technological solution for the delivery of drugs is nanotechnology. Nanotechnology is defined by Farokhzad and Langer as "the engineering and manufacturing of materials at the atomic and molecular scale" (Farokhzad and Langer, 2009). The structures range in size of 1 to 100 nm in at least one dimension. Nanotechnology itself is a very diverse field ranging in applications from medicine, electronics, biomaterials and energy production (NNI, 2008). Nanotechnology became popular in the 1980s although the concept had been discovered earlier. The reason being the invention of the scanning tunneling microscope in 1981 by Gerd Binning and Heinrich Rohrer at IBM Zurich Research Laboratory along with fullerenes in 1985 by Harry Kroto, Richard Smalley, and Robert Curl gave nanotechnology the potential to create functional systems at a molecular scale (Ratner et al., 2003). The possibilities are truly infinite.

2.2 <u>**Nanotechnology Based Targeted Drug Delivery**</u>

The awareness of nanotechnology in the field of medicine grew in the early 2000s with programs promoting and funding this research ((Ratner et al., 2003). One of the major research programs is the National Nanotechnology Initiative. The research includes developing new approaches to drug delivery and targeting, and visualization in a surgical field. Drug delivery and targeting includes using nanotechnology to improve delivery of non-soluble drugs, target specific cell or tissues, transcytosis of drugs across epithelial and endothelial barriers, and co-delivery of two or more drugs for combination therapy (Farokhzad and Langer, 2009; Ruoslahti et al., 2010; Hawkins et al., 2008; Vladimir, 2006). Nanotechnology can increase visualization of the sites of drug delivery by combining therapeutics with imaging agents and repair capabilities (Farokhzad and Langer, 2009). Nanosized objects are particularly attractive delivery vehicles as they combine properties important for targeting with a small particle size giving them a still reasonably high mobility/diffusivity to penetrate tissue barriers. Therefore, the application of nanotechnology drug delivery systems in clinical medicine, especially in cancer, has become the way to combine the delivery of hydrophobic agents to specific regions and reduce the dose thereby limiting toxicities caused by nonspecific drugs (Hawkins et al., 2008).

2.3 <u>**Taxanes as Antitumor Drugs**</u>

Important classes of antitumor agents that are used in solvent-based delivery vehicles are taxanes. Two in particular, paclitaxel and docetaxel, have been established as the main treatments for early stage and advanced breast cancer (Farokhzad and Langer, 2009). Clinical advances in the use of taxanes, however, have been limited by their chemical formulation as they are highly hydrophobic molecules. To overcome the poor water solubility, lipid-based solvents

are used as solvent vehicles. These solvents are extremely effective yet solvent based formulations have been associated with detrimental toxicities including hypersensitivity reactions, neutropenia, and neuropathy (Hawkins et al., 2008). A solution to these adverse side effects is the development of new delivery vehicles based on nanotechnology to improve and increase antitumor activity while reducing toxicity. Some of the developed and studied novel formulations for the delivery of taxanes include proteins, liposomes, polymers (L-γ-glutamyl-glutamine), and peptide conjugates (Vladimir, 2006).

Liposomes were among the first nanotechnology drug delivery systems (Farokhzad and Langer, 2009; Vladimir, 2006). These lipid vesicles have been studied since the 1960s and are considered to be one of two dominant classes of conjugates used today (Farokhzad and Langer, 2009). There are many studies involving the use of liposomes for the delivery of drugs. One particular group, Zhijun Yang from Wuhan Bioengineering Institute in China, has been studying the development of an *in situ* gel system consisting of liposome-paclitaxel conjugate (PTX) within a hydrogel, Pluronic F127, for drug delivery (Nie et al., 2011). In these studies, they utilized dialysis membranes and membrane-less diffusion to determine the rate of drug release. Their results showed that the thermo-sensitive hydrogel containing the liposome-paclitaxel conjugate had the longest release period. Therefore, this formulation has the potential of being a good system for treatment of local cancers.

The other dominant class of conjugates for drug delivery is polymers (Farokhzad and Langer, 2009). Polymers are used as either polymeric PTX micelles or polymer-PTX conjugates (Van et al., 2010; Yang et al., 2011). Yu et al. combined both types of polymers into one

nanoparticle delivery system that contained both entrapped PTX and conjugated PTX. The purpose of the study was to establish the efficacy of this novel formulation: PTX-loaded PGG-PTX compared to PTX and saline. They concluded that while PTX-loaded PGG-PTX induced significant tumor regression, it also showed signs of increased toxicity in mice with tumors as opposed to healthy ones.

2.4   Abraxane: Nanoparticle Based Formulation of Paclitaxel

Celgene (previously known as Abraxis Bioscience, Inc) developed a nanoparticle based drug formulation named Abraxane using their proprietary nanoparticle albumin-bound technology. Abraxane is composed of albumin and paclitaxel and is a chemotherapeutic drug used for the treatment of breast cancer. Abraxane is an albumin-bound 130-nm particle formulation of paclitaxel. It is used as a colloidal suspension derived from the lyophilized formulation of paclitaxel and human serum albumin diluted in physiological saline solution. Human serum albumin stabilizes the drug particle at an average primary particle size of 130 nm which prevents any risk of capillary obstruction. Abraxane is the first protein engineered nanoparticle on the market that has been approved for treatment of breast cancer in patients that have either failed combination chemotherapy for metastatic disease or suffered a relapse within 6 months of adjuvant chemotherapy (Hawkins et al., 2008).

2.5   Paclitaxel: Discovery and Use of a Potent Anticancer Agent

Paclitaxel is the active ingredient in Abraxane and mediates the cytotoxic effects of the drug. Paclitaxel was discovered in a National Cancer Institute research program in which thousands of plants were collected and screened for anticancer activity. One of these samples,

the bark of a Pacific *yew Taxus brevifolia*, was found to have such anticancer activity (Wani et al., 1971; Schiff et al., 1979, Rowinsky and Donehower, 1995; Sparreboom et al., 2005; Desai et al., 2006). Fractionation and isolation of the active ingredient of this sample was performed in the laboratory of Monroe E. Wall in Research Triangle Park, North Carolina. In 1971, Wall and colleague Wani identified paclitaxel as the active constituent and published its chemical structure (Wani et al., 1971). In 1979 Susan B. Horwitz, a molecular pharmacologist at Einstein College of Medicine, identified paclitaxel's mechanism of action which involved the stabilization of microtubules creating further interest from researchers (Schiff et al., 1979). The National Cancer Institute began clinical trials in 1984 which showed antitumor effects in patients which brought about a limited supply of the drug (Rowinsky and Donehower, 1995). These trials required the extraction of thousands of pounds of bark to yield the required amount of drug to treat one patient which was later resolved by the development of a synthetic form of the compound by Bristol-Myers Squibb (Rowinsky and Donehower, 1995).

Paclitaxel is used for the treatment of various cancers including breast cancer, non-small cell lung carcinoma, ovarian cancer, head and neck cancer, and advanced forms of Kaposi's sarcoma (Rowinsky and Donehower, 1995). The most common characteristic of cancer cells is their ability to replicate in an uncontrolled manner causing extensive restructuring of the cell's cytoskeleton which is effectively inhibited by paclitaxel (WHO, 2011; ACS, 2010a). The primary mode of action is of this anti-microtubule agent is to promote the assembly of microtubules from tubulin dimers and then stabilizes microtubules to prevent depolarization (Schiff et al., 1979, Rowinsky and Donehower, 1995), thereby inhibiting normal cell replication and the proliferation of cancer cells (Rowinsky and Donehower, 1995).

Standard paclitaxel (paclitaxel-Cremophor formulation) is generally administered intravenously at a dose of 175mg/m2 for over 3 hours to patients who must be pre-medicated with corticosteroids or antihistamines (Hawkins et al., 2008; Rowinsky and Donehower, 1995). As a result, patients suffer from numerous side effects associated with pre-medication and toxic side effects. The main side effects include neutropenia, alopecia, peripheral neuropathy, nausea and diarrhea among others (Hawkins et al., 2008). These side effects reduce the quality of life of patients, thus creating the need for new treatments that will increase the quality of life and most importantly have potentially more effective outcome in destroying the cancer cells.

Celgene used their newly developed nab (nanoparticle albumin-bound) technology to develop such a drug. As stated earlier, Abraxane is a 130 nm albumin-bound formulation of paclitaxel and was created to replace and/or serve as an alternate treatment to paclitaxel-Cremophor conjugate (Hawkins et al., 2008; Sparreboom et al., 2005; Gradishar et al., 2005; Desai et al., 2006). After several preliminary studies, the Food and Drug administration approved the use of Abraxane in 2005 for clinical trials for treatment for breast cancer (Hawkins et al., 2008; Celgene, 2010). Preliminary studies showed Abraxane had a higher volume of distribution and plasma clearance than Taxol which increased its therapeutic effectiveness (Gradishar et al., 2005).

Phase III clinical trials verified the results from previous studies which showed greater efficacy and reduced toxicity of Abraxane compared with standard paclitaxel (Gradishar et al., 2005). Abraxane is given as 30 minute IV infusion every 3 weeks at a dose of 260 mg/m2, no

pre-medication is required (Sparreboom et al., 2005; Gradishar et al., 2005; Desai et al., 2006). However, there are still side effects associated with Abraxane as you would expect with any drug and especially a cytotoxic chemotherapeutic drug. These side effects are the same as those observed with Taxol, primarily alopecia, sensory neuropathy, nausea and diarrhea (Gradishar et al., 2005). In spite of these side effects, patients treated with Abraxane do not endure the detrimental effects associated with pre-medication and have shorter treatment regimens with positive results in comparison to standard paclitaxel (Sparreboom et al., 2005; Gradishar et al., 2005; Desai et al., 2006).

2.6   Abraxane: Improved Bioavailability Due to Increased Transcytosis

The drug formulation of Abraxane as an albumin-bound paclitaxel conjugate was designed to increase the bioavailability and efficacy and reduce the toxicity associated with standard paclitaxel (Sparreboom et al., 2005; Gradishar et al., 2005; Desai et al., 2006). This combination was proposed to allow albumin to play a major role in endothelial binding and transcytosis, enhancing the intratumor delivery of Abraxane. Transcytosis, or transcellular transport, occurs via caveolae in endothelial cells (Desai et al., 2006; Minshall et al., 2002; Minshall et al., 2003; Hu et al., 2008). Caveolae are small cholesterol-enriched membrane microdomains that contain the protein caveolin-1. The caveolae mediated transport pathway is thought to be the primary pathway of albumin transport across the endothelium in the basal or non-injured state (Desai et al., 2006; Minshall et al., 2002; Minshall et al., 2003; Hu et al., 2008). As described by Minshall et al, the transport process begins with the binding of albumin to albumin binding proteins, primarily the 60-kDa albumin binding glycoprotein gp60. Binding of albumin and gp60 activates albumin endocytosis via caveolae and thus the release of vesicles

from membrane. Upon albumin binding to gp60, Src protein tyrosine kinase is activated and caveolin-1 becomes phosphorylated, triggering the release of caveolae, i.e., internalization. Finally through currently undefined mechanisms, caveolae-mediated transport shuttles albumin across the endothelial barrier, from the blood to the tissue (Minshall et al., 2002; Minshall et al., 2003; Hu et al., 2008).

In this study, we investigate the uptake of Abraxane using murine models in which caveolin-1 was either present (WT) or absent (KO). We propose that the absence of the caveolin-1 gene will greatly reduce the bioavailability and efficacy of Abraxane supporting the hypothesis that caveolae-mediated transport is required for Abraxane uptake *in vivo*. The results obtained from this study will help answer the question whether caveolae mediates or facilitates the uptake of the albumin-bound paclitaxel. Overall, the study will provide useful information on this newly developed drug and hopefully in the near future will help to alleviate the global burden of breast cancer.

3. MATERIALS AND METHODS

3.1   Chemicals and Reagents

Abraxane was obtained from Celgene, previously known as Abraxis Bioscience, LLC, (Marina del Rey, CA). Abraxane was reconstituted in 0.9% NaCl. Although Abraxane is a very stable drug, it was prepared fresh on the day of experiment to ensure all mice were treated similarly. Abraxane was administered at a dose of 150 mg/kg, which contains an equivalent of 15 mg paclitaxel.

Paclitaxel was purchased from ChemieTek, LLC (Indianapolis, IN). Paclitaxel was first reconstituted in 100% DMSO (Sigma Aldrich, St. Louis, MO) due to its low aqueous solubility, and thereafter combined in a 1% albumin (Sigma Aldrich, St. Louis, MO) solution for administration. Paclitaxel was also prepared fresh on the day of the experiment and given at a dose of 15 mg/kg.

3.2   Liquid Chromatography Mass Spectrometry

Paclitaxel-d5 (Deuterated internal standard) used for LCMS was purchased from Toronto Research Chemicals, Inc (Toronto, ON Canada). An HPLC column used for LCMS measurements was purchased from Varian (Agilent Technologies, Santa Clara, CA). The LC/MS/MS system used was a Waters 2795 Separations Module and Micromass Quattro micro API Mass Spectrometer with MassLynx 4.1 Software (Waters Corporation, Milford, MA).

3.3   <u>Animals</u>

B6/129SF2/J (wild-type) and Caveolin-1$^{-/-}$ (knock out) mice originally purchased from Jackson Laboratories (Sacramento, CA) and presently bred in-house were used in the study. They were housed in the College of Medicine Research Facility which is maintained at 70C with 12 hour light cycles as outlined by the Animal Welfare Act. Only female mice of 8-12 weeks of age were used.

3.4   <u>Animal Experiments</u>

Mice were weighed and anesthetized with isoflurane (inhalation anesthetic). They were then shaved and prepared for surgery using proper aseptic techniques. A single bolus infusion of Abraxane or paclitaxel via the external jugular vein was administered at a dose of 150 mg/kg or 15 mg/kg respectively. Mice were then euthanized at various time points (0, 1, 4, 12 and 24 hour) and tissues of interest (plasma, lung, liver, kidney, breast and brain) were collected. Blood was cleared from mice (whole body) after a sample blood aliquot was acquired for separation of the plasma which was assayed for drug concentration in each mouse. Tissues were stored at -80C until they were processed and analyzed for total and unbound paclitaxel using LCMSMS.

3.5   <u>External Jugular Vein Injection & Tissue Collection</u>

Mice were weighed and anesthetized with 1.5-2 % isoflurane (inhalation anesthetic) during the duration of surgery which was about 10 minutes. The external jugular vein was exposed when a surgical plane of anesthesia was reached, which was determined by the lack of response to paw or tail pinch and eye reflex. The ventral surface of the neck area, off midline and just above the external jugular vein, was shaved, cleaned, and de-germed using a providone-

iodine solution. Additionally, the surgical site was isolated using sterile drapping providing a working surface for sterile (autoclaved) surgical instruments. A small incision (approximately 3 mm) was be made for access and cannulation of the right external jugular using a catheter (ID .22mm, OD .61mm). The vein was then separated from surrounding tissue using blunt curved forceps and 2 ties (6-0 silk suture), proximal and distal, placed around the vessel, which was ligated by tying the distal suture. The distal suture was retracted and a small incision was made in the vessel to allow the passage of the catheter. The catheter was inserted past the proximal suture, which as then tied to secure the catheter in place for the single bolus infusion of Abraxane or Paclitaxel (control). Finally the catheter was removed and the wound closed using 6-0 prolene suture. The wound was irrigated with 100 mcl 2% Lidocaine HCl.

For tissue collection, the mice were anesthetized with 1.5-2 % isoflurane (inhalation anesthetic) and an incision to the abdomen was performed for the procurement of tissues only after a surgical plane of anesthesia was reached, as determined by the lack of response to paw or tail pinch and eye reflex. 50 mcl of 1000 units heparin was injected initially into the inferior vena cava and allowed to circulate for 1 minute. About 300-400 mcl blood sample was collected and placed on ice until centrifugation to isolate plasma, which was then stored at -80C. Blood was cleared from the whole mouse via inferior vena cava catheter infused with 10 ml of sterile 0.9% NaCl. Once the blood was cleared from the body, tissues of interest (lung, kidney, liver, breast, and brain) were immediately collected and flash frozen in liquid N2 and then held at -80C until analysis.

3.6   <u>**Liquid Chromatography Mass Spectrometry Measurements**</u>

Standards and quality control samples were prepared by serial dilution to establish a standard curve for plasma and each tissue sample. Paclitaxel is very stable, and therefore freeze-thaw cycle had no effect on the measurements but all samples were always kept on ice and treated equally.

Plasma samples were thawed on ice and a 100 µl aliquot was placed in a 1.5ml tube. 400 µl of acetonitrile containing the internal standard was added to each tube and vortexed for 1 minute. Samples were centrifuged for 10 minutes at 10,000g and the supernatant was then placed in a fresh tube where it was evaporated with liquid N2. The sample was then reconstituted with the mobile phase buffer and centrifuged again for 10 minutes at 10,000g. The supernatant was transferred into a glass tube from which the supernatant was injected into the LC/MS/MS system for sample analysis.

All tissues were weighed and homogenized in 3 volumes of 1X PBS (without calcium and magnesium) per gram of tissue. The samples were homogenized on ice using a polytron tissue homogenizer (Kinematica Polytron PT 3000) until a homogenous state was reached. The homogenizing probes was washed thoroughly between homogenizations to ensure there was no cross contamination between samples. Three different washes at different concentrations were used between samples.   Wash #1 consisted of 84:10:5:1 Methanol: Water: Acetonitrile: Isopropanol; wash #2 consisted of 50:50 Methanol: Water; wash #3 consisted of 20:80 Methanol: Water combination. It is critical to wash three times between each sample and in this particular order to prevent any contamination of the next sample. An aliquot of 100 µl of sample

homogenate was placed into a 96 well plate along with the proper quality control samples and matrix blanks. Then, 400 µl of acetonitrile containing the internal standard was added to each sample and vortexed for 1 minute. Samples were centrifuged for 10 minutes at 10,000g and the supernatant was then injected into the LC/MS/MS system for sample analysis.

The reported concentration values from each sample were exported into an Excel file for calculations and interpretation. Statistical analysis was performed using the program GraphPad Prism. Analysis of variance and Student's t-test were used to determine statistical significance of the data.
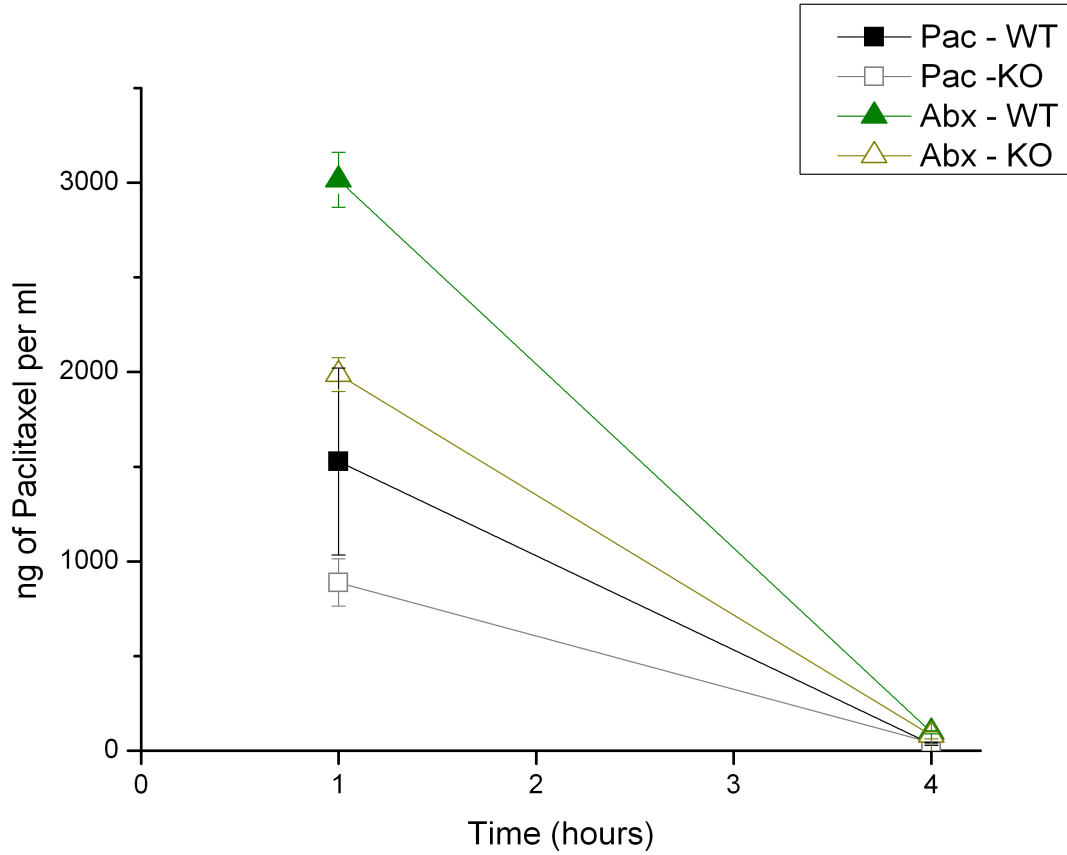
4. RESULTS

Figure 1. Plasma levels of paclitaxel and abraxane.

After intravenous administration of either Paclitaxel or Abraxane, statistical differences in plasma levels are observed at 1 hour between Paclitaxel and Abraxane. The level of Abraxane is 2 times higher than of Paclitaxel and also higher in B6/129SF2/J (wild-type) mice. Two-tailed unpaired t-test gave a p value of 0.005. Two-way analysis of variance for Abraxane in WT versus Caveolin-1 [-/-] (KO) is significant. The p value is 0.0009.
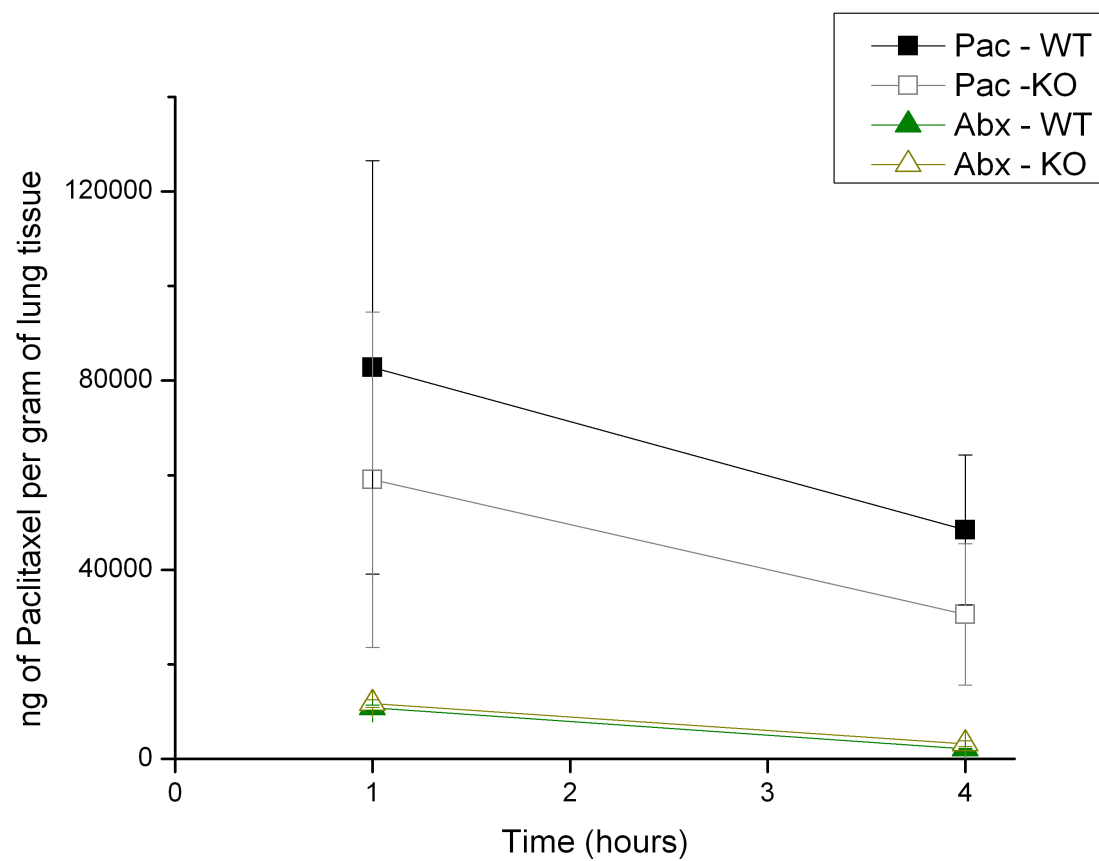
Figure 2. Drug accumulation in lung tissue.

Paclitaxel accumulation in the lung was not statistically different from Abraxane levels in either the WT and KO mice (n=4).
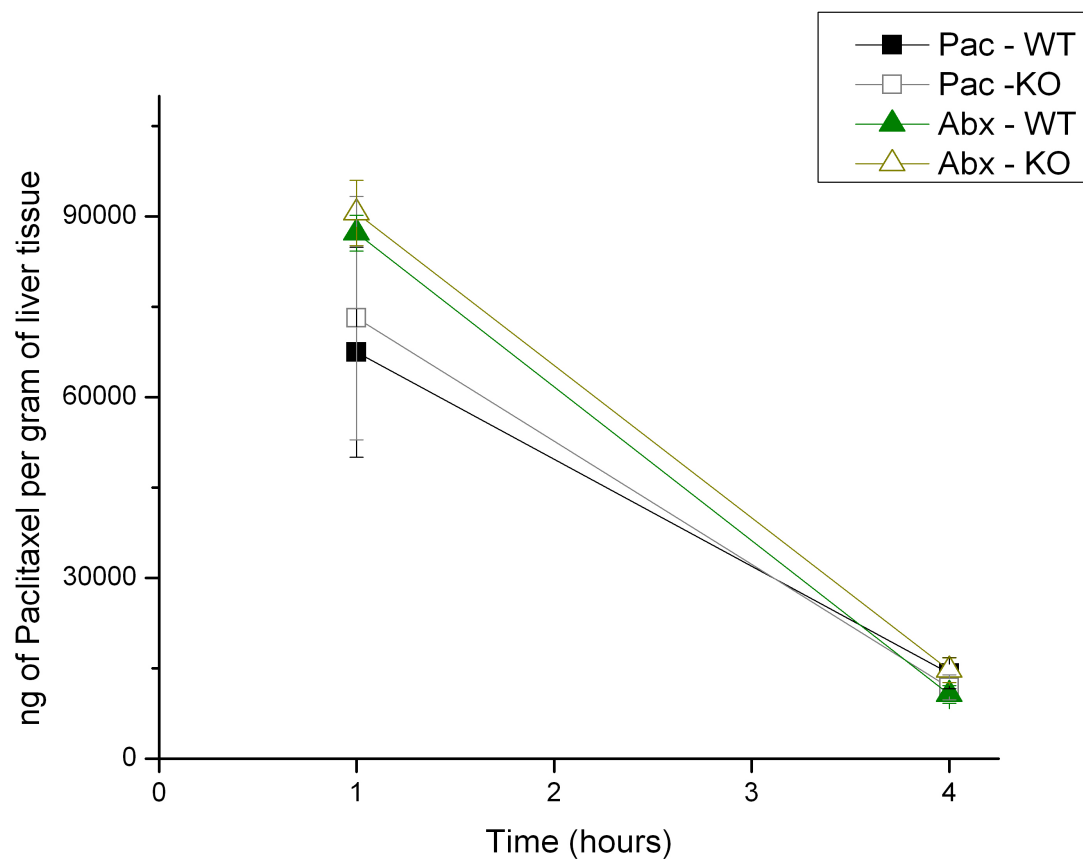
Figure 3. Liver.

Although there is a decrease of both drugs in the liver with time, there were no significant differences between both WT and KO for either drug.
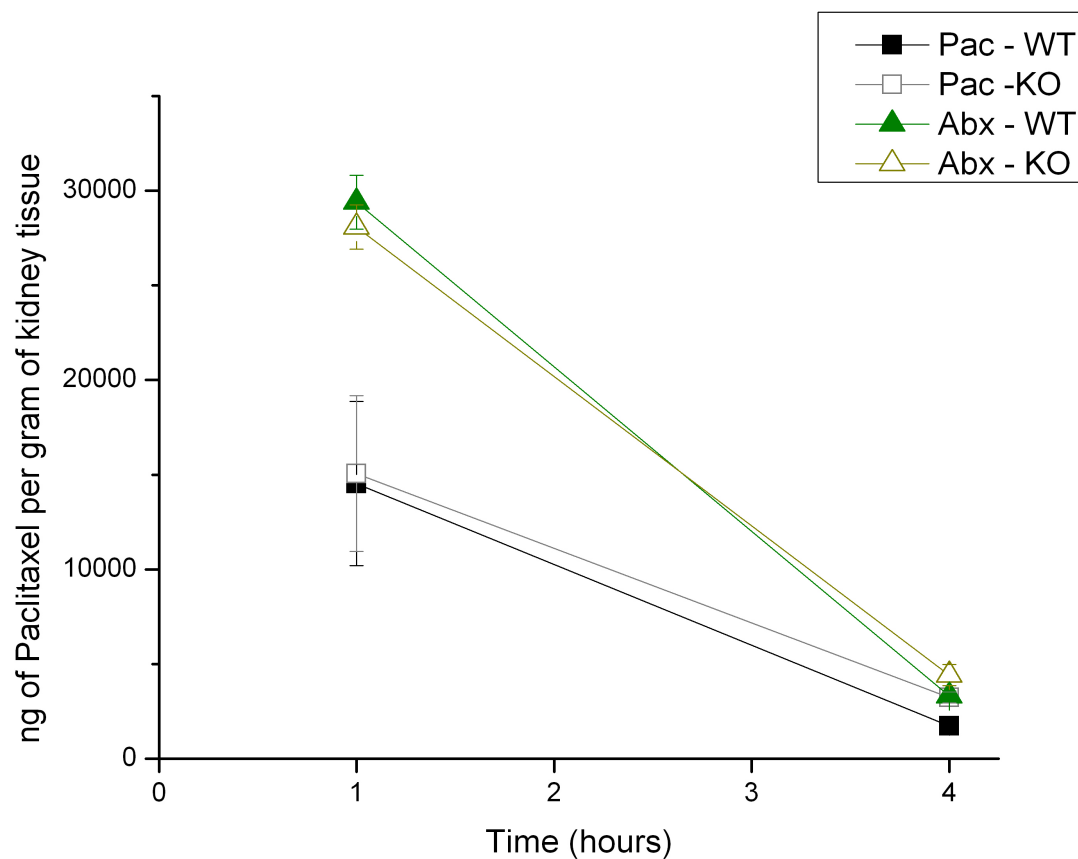
Figure 4. Kidney.

Accumulation in the kidney is much higher for Abraxane for both WT and KO mice. Accumulation of paclitaxel and Abraxane in WT mice at 1 hour and 4 hour are significant with p values 0.0173 and 0.0105 respectively. For KO mice, there was significance between drugs at 1 hour with p value of 0.0227 but there was no difference at 4 hours. The accumulation of paclitaxel in WT and KO mice was not significantly different at 1 hour but was significant at 4 hour with p value 0.0153, while Abraxane accumulation was not different for either WT or KO at both 1 hour and 4 hour.
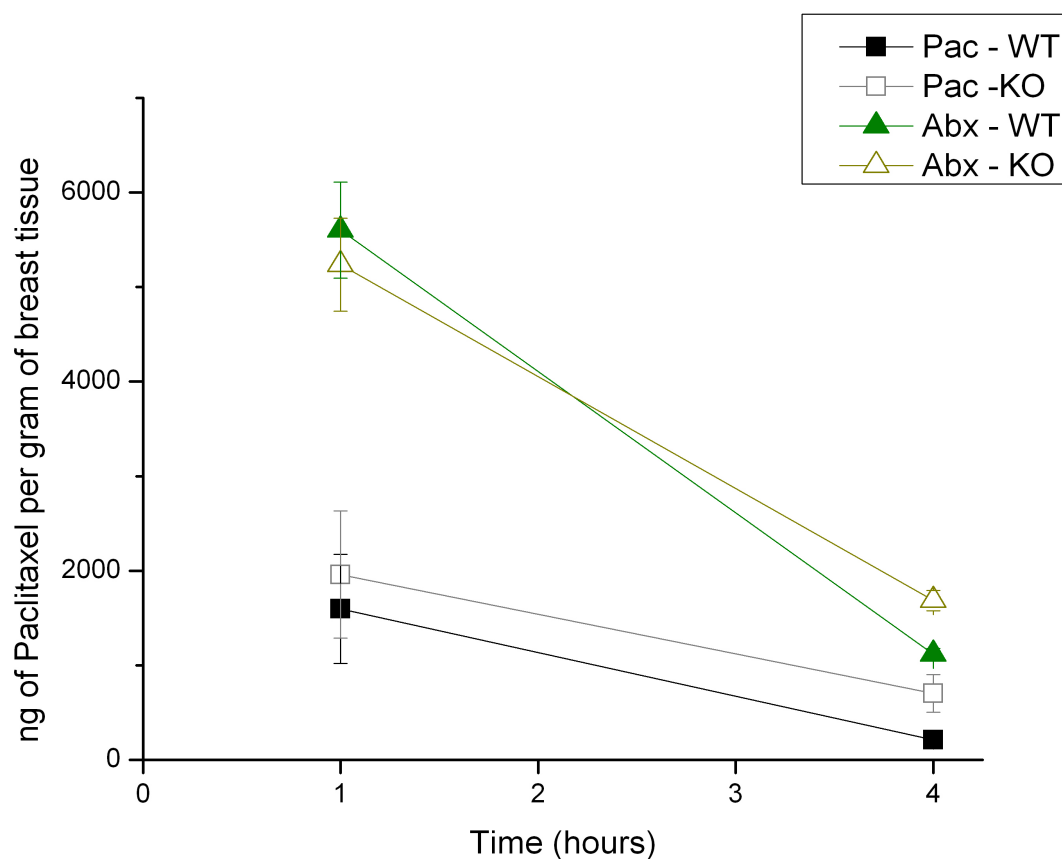
Figure 5. Breast.

Two way analysis of variance of Paclitaxel and Abraxane measured in breast tissue showed a significant difference in accumulation in WT mice and also over time. The p values are 0.0020 and 0.0002 respectively. The same was observed for KO mice at both 1 hour and 4 hour and their p values are 0.0070 and 0.0049 respectively. The accumulation of paclitaxel in WT and KO mouse breast tissue was not significant, but in mice injected with Abraxane, there was significantly greater accumulation at 4 hours ($p < 0.005$) in KO mice compared to WT.
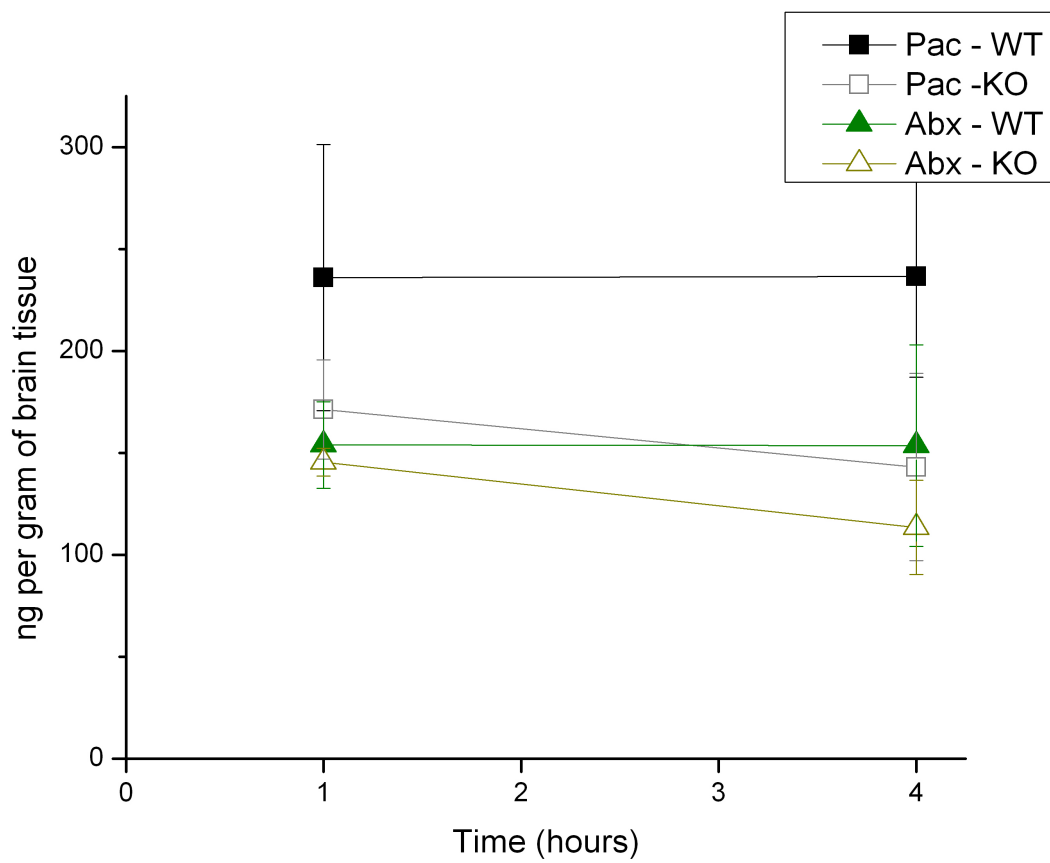
Figure 6. Brain.

There was very little Paclitaxel or Abraxane that accumulated in the WT and KO mice and values were not significantly different between groups.

Figure 7. Biodistribution.

There was a difference in biodistribution between Paclitaxel and Abraxane in both WT and KO mice.   The order of highest tissue accumulation for paclitaxel was lung>liver>kidney>breast>brain and for Abraxane it was liver>kidney>lung>breast>brain. The tissue levels decreased over time for both paclitaxel and Abraxane but the order remained the same.

5. DISCUSSION

The purpose of the study was to demonstrate whether the uptake of Abraxane was mediated specifically by the caveolae-dependent transport pathway using murine models in which c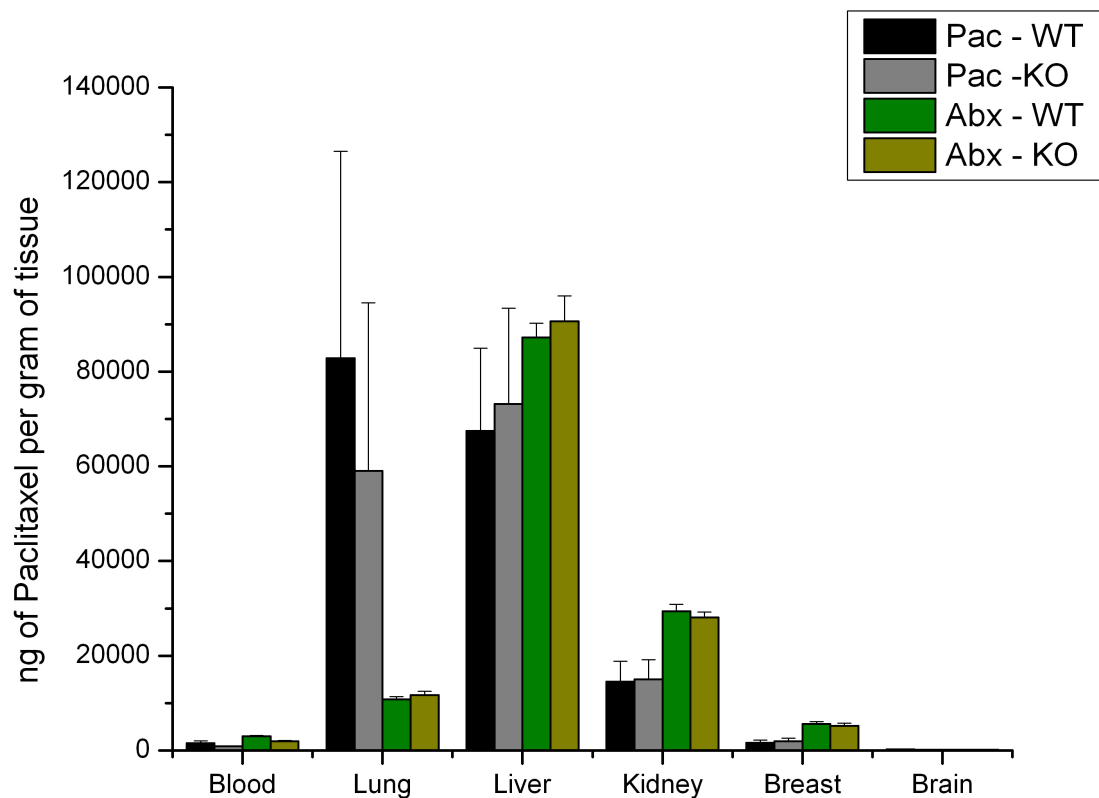aveolin-1 was either present (WT) or absent (KO). We proposed that absence of the caveolin-1 gene would greatly reduce the bioavailability and efficacy of Abraxane uptake, supporting the hypothesis that caveolae-mediated transport is required for Abraxane uptake *in vivo*.

B6129SF2/J (wild-type) and Caveolin-1 -/- (knock out) mice were injected with either standard paclitaxel or Abraxane at equal doses of active compound and tissues were then procured at different times in order to determine the level of drug accumulation in each mouse strain for the specified tissues. Based on the results obtained, it appears that caveolin-1 is not required for Abraxane uptake. Abraxane accumulated in both WT and KO mice in the same manner and was not statistically different between the two groups. Regardless, there is some correlation between the results from this study and previous studies performed by Desai et al. (Sparreboom et al., 2005). Both studies showed Abraxane levels were initially higher than paclitaxel, suggesting longer circulation times and bioavailability for the former.

Plasma levels in Abraxane were two fold greater than that of paclitaxel at 1 hour after i.v. injection. Therefore Abraxane is metabolized and cleared at a slower rate allowing for longer circulation time and distribution, consistent with clinically observed longer circulation times and bioavailability. Also, the level of Abraxane was higher in WT mice compared to KO. Paclitaxel accumulated more than Abraxane in lung tissue with no difference between WT and KO. One

explanation for this observation is the fact that paclitaxel is a small molecule and is unconjugated, and therefore it can enter more easily than Abraxane which is conjugated and has a significantly higher diffusion coefficient. One can say that Abraxane is not necessarily the choice of treatment for lung diseases. The data on liver tissues does not show any difference between the accumulation of both drugs in WT and KO mice suggesting they are both metabolized and cleared within 4 hours. Abraxane accumulates twice more effectively than paclitaxel in the kidney for both WT and KO resulting in no significant differences between both drugs but none between species. However, there is somewhat of an indication that caveolin-1 plays a role in the accumulation of paclitaxel in the kidney. This observation suggests the possibility of prescribing paclitaxel for treatment of kidney disease. The brain had the lowest accumulation of both Abraxane and paclitaxel in both WT and KO mice, and these differences were not significant as reported previously by Desai et al. 2005. Regardless of the low levels observed for both drugs on the brain tissue, the likelihood of drug toxicity due to exposure time over 4 hours remains. There is not yet enough information to know whether harmful effects of paclitaxel or Abraxane might be observed in the brain. The low levels of accumulation of both drugs suggest neither drug crossed the blood-brain barrier within 4 hours. Finally, in breast tissue (tissue of interest because Abraxane is used for treatment for breast cancer), the accumulation of Abraxane was 3 fold higher than paclitaxel and more in KO mice than WT. This suggests that albumin facilitated the internalization of the paclitaxel conjugate into breast tissue and thus caveolin-1 may be a key player in this mechanism. Abraxane is the better choice for treatment for diseases of the breast. On the other hand, the fact that Abraxane was also found in high levels in WT mice implies the possible toxicity of the drug in normal breast tissue.

An important conclusion from this study is that Abraxane is taken up by normal healthy tissues, primarily kidney and breast. Abraxane is used currently used for the treatment of breast cancer, and this study supports the notion that Abraxane is taken up significantly in breast tissue. This scheme has been supported by Sugahara et al. This study uses the combined administration of Abraxane and a tumor-penetrating peptide (iRGD) (Sugahara et al., 2010).   They demonstrated this pairing increases uptake of Abraxane by 12 fold in breast tumors compared to tumors in other tissues, but was not significantly different than Abraxane alone (Sugahara et al., 2010). This evidence supports the use of Abraxane as an efficient and specific treatment for breast cancer. On the down-side, normal tissues were used for this study and we do not want Abraxane to accumulate into normal breast tissues in patients with breast cancer, but rather only in breast tumors, creating a key concern.

Abraxane was designed to reduce the side effects associated with the premedication and formulation of Cremophor-EL (current treatment for several cancers). The albumin-paclitaxel conjugate has in fact eliminated any premedication and has shortened the duration of treatment while reducing some of the side effects caused by Cremophor-EL. Uptake of Abraxane in healthy tissues as shown in our study suggests it is possible that unwanted toxic side effects may be attributed to the specific action of Abraxane. The amount and severity of these possible toxic effects cannot be determined at this point except to acknowledge the fact that there may not be much distinction between healthy and cancerous cells with regards to Abraxane.

The pathway in which Abraxane is taken up by tissues in vivo cannot be determined from this study and remains unknown. More studies need to be performed to determine whether

Abraxane is specifically internalized and transported by vascular endothelial cells in vivo, as proposed by the manufacturer, as apposed to simple diffusion of free paclitaxel from the blood into tissues. This study suggests that, at least in normal mice, uptake and transport of Abraxane does not appear to be mediated via caveolae-dependent mechanisms.

One potential explanation for the observations made in this study relates to the vascular "leakiness" of caveolin-1 null mice, which may in fact have increased microvascular permeability due to the increased incidence of open inter-endothelial cell junctions (Carver and Schnitzer, 2003). The KO mice would still be able to transport molecules such as albumin into the tissue through the paracellular pathway via these open intracellular junctions. This compensatory mechanism may have masked any differences that might have been observed otherwise. For example, in WT mice there should be some uptake of intact Abraxane by caveolae and also some diffusion of the free drug, paclitaxel, depending on the tissue bed, whereas nearly equivalent levels of paclitaxel or Abraxane (albumin conjugated or otherwise) may have diffused through the open junctions in the Cav1 KO mice.

As alluded to above, another possible interpretation of these data could be that Abraxane conjugation with albumin may not be as stable *in vivo* in mice as it is has been shown to be *in vitro*. Abraxane uptake by tumor endothelial cells may be greater than normal ECs, although this was not measured in the present study. We measured the level of total paclitaxel (both bound and unbound paclitaxel) in the plasma and tissues. In the study from Desai et al. (2005) compared the biodistribution of paclitaxel in tissues using radio-labeled Taxol and Abraxane. They reported the values for lung tissue that were 3.6-fold higher for the Taxol formulation

compared to Abraxane and no significant differences were observed for either drug formulation for any other tissues (i.e., Cremophor vs. albumin) (Sparreboom et al., 2005). Therefore, the Abraxane formulation used clinically is basically the same as paclitaxel mixed with 1% albumin and that the "nanoparticle" may not be very stable in vivo. Thus, uptake of free drug may be what we measured in this experiment.

One of the limitations of the study is that we did not collect blood and tissue samples at 15, 30, or 45 minutes after injection which would have provided more insight into the our understanding of the distribution of paclitaxel vs. Abraxane. Most of the drug was eliminated from the tissues within 4 hours, therefore, the data for the 12 and 24 hour time points was excluded from the graphics presented. Plasma levels showed that the majority of the drug was taken up by tissues within the first hour and thus a more complete picture of the uptake kinetics requires additional sampling at less than hour after injection. Also, the control used for the study was paclitaxel in a 1% albumin solution and not the Cremophor-EL formulation used clinically (Taxol) and in the majority of other published animal studies.

Future studies could also employ siRNA to knockdown the caveolin-1 gene in wild-type mice as a means of assessing the role of caveolae in Abraxane up take in vivo. This would resolve the issues associated with the Cav-1 knockout mice which are known to have a abnormal and "leaky" vasculature (Razzani et al., 2001; Schubert et al., 2002; Maniatis et al., 2008) which may have influenced in the results. Another possible study could include the infusion of filipin or methyl-β-cyclodextrin to flatten caveolae and thereby reduce/inhibit the intracellular and transcellular transport of albumin across the endothelium (Vogel et al., 2001). This may be

helpful in distinguishing whether transport is mediated by the proposed caveolae-mediated uptake mechanism.

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