DEVELOPMENT OF COLCHICINE-LOADED PEGYLATED CATIONIC LIPOSOMES AGAINST LEWIS LUNG CARCINOMA

MS Thesis presented

by

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ABSTRACT

The role of antitubulin drugs as vascular disrupting agents against cancer has been widely recognized. These agents bring about tumor ischemia and necrosis caused by microtubule disruption, alterations in cytoskeleton function of endothelial cells lining microvasculature, change in cell structure and in blood flow dynamics resulting in complete vascular shutdown in solid tumors. The drug colchicine (an alkaloid) is currently used to treat gouty arthritis but was initially shown to exhibit significant tumor vascular disrupting effects. Colchicine acts by depolymerizing microtubules but despite its impressive therapeutic profile as a vascular disrupting agent, it is too toxic to administer to cancer patients by IV route. We therefore developed PEGylated cationic liposomes (PCLs) loaded with colchicine to enhance tumor vascular-specific uptake of the drug and therefore reduce unwanted side-effects. LLC-Lewis lung Carcinoma, MS1-VEGF- endothelial cell lines were used as in vitro models. Colchicine-PCL preparations varied from 3 to 10 mole% drug and their like particle size and zeta potential were determined using a Zeta Particle Size Analyzer. The incorporation of colchicine in PCLs was evaluated by UV microplate reader and reverse phase HPLC analysis. The cytotoxicity profile was established by Sulforhodamine B (SRB) assay using a fluorescence microplate reader. Qualitative and quantitative analysis of the cytoskeleton was performed on each cell line using FITC-labeled β-tubulin antibody and the mean area of cytoskeleton and nucleus per cell was determined with free colchicine and colchicine-loaded PCLs using a fluorescence microscope. The extent of cytoskeletal disruption in each case was quantified using BIOQUANT software.
Thesis title: Development of Colchicine-loaded Pegylated Cationic Liposomes against Lewis Lung Carcinoma

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Program: Pharmaceutical Sciences

Approval for thesis requirements of the Masters of Science Degree in Pharmaceutical Sciences

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Table 1 – Physicochemical properties of colchicine loaded pegylated cationic liposomes
ABBREVIATIONS

DOPC - 1,2-Dioleoyl-\textit{sn}-Glycero-3-Phosphocholine

DOTAP - 1,2-Dioleoyl-3-Trimethylammonium-Propane

DOPE - L-a-Dioleoyl Phosphatidylethanolamine

PEG\textsubscript{5000} - PolyEthyleneGlycol (MW-5000)

TC – Tubulin-Colchicine

GTP – Guanosine Tri Phosphate

GDP-Pi – Guanosine Di Phosphate – Inorganic phosphate

TNF – Tumor Necrosis Factor

IL-1 – Interleukin – 1

5-FU – 5- Flurouracil

PCL – Pegylated Cationic Liposomes

DAPI – 4, 6-diamidino-2-phenylindole

FITC – Fluorescein Isothiocyanate

TCA – Tricholo Acetic Acid

DIC – Differential Interference Contrast

BSA – Bovine Serum Albumin

LLC – Lewis Lung Carcinoma, Murine cell line

MS1-VEGF – Murine Pancreatic Endothelial Cell line

SCLC – Small Cell Lung Carcinoma

NSCLC – Non small Cell Lung Carcinoma

SRB – Sulforhodamine B

VTAs – Vascular Targeting Agents
1. Background and Significance

Cancer is defined as any of the various malignant neoplasms characterized by the proliferation of anaplastic cells that tend to invade surrounding tissue and metastasize to new anatomical sites (1). There are more than one hundred different types of cancer and subtypes of tumors that can arise from every organ (2). Cancer is characterized by six essential acquired capabilities in cell physiology: a) self sufficiency in growth-inhibitory signals, b) evasion of programmed cell death (apoptosis), c) limitless replicative potential, d) sustained angiogenesis, e) tissue invasion and f) metastasis (2).

1.1 Lung Cancer

Lung cancer is the leading cause of cancer deaths in the USA and presently contributes to 31% and 29% of all cancer deaths in males and females respectively (3). Deaths from lung cancer alone outnumber the total number of deaths from breast, prostate and colon cancers combined (3). Extensive research has been carried out to identify and monitor tumor markers for the diagnosis at early stages of cancer disease. The etiology of the exudative pleural effusion is an excellent technique to diagnose malignant growths in the lung. The positivity of mucinous marker (CA-15 3) and thymidine kinase marker (TK) in the pleural exudates strongly indicates the presence of malignant exudates (4). The World Health Organization specifies different types of lung cancer depending on the kind of cells involved (5). Non-small cell lung cancer (NSCLC) and Small cell lung cancer (SCLC) make up over 90 percent of all lung cancer cases. Non-small cell lung cancer represents about 80% of the cases and is characterized by slow metastasis, which is why these tumors are mostly confined to one or two lobes within the lung when first
diagnosed. Based on type of tissue affected, NSCLC is further classified into squamous cell carcinoma (outermost layer), adenocarcinoma (mucous cells), large cell carcinoma comprising two new variant tumors, large cell neuroendocrine tumors and basloid tumors (at site of entry of bronchiolar tubes into lungs) and bronchioalveolar carcinoma (the bronchi and alveoli) (5). SCLC comprises 20% of all cases, exhibits aggressive metastasis and is diagnosed only after extensive spread of the disease. Depending upon the stage at which the cancer is diagnosed SCLC is classified as limited disease and extensive disease. Progression of disease to the mediastenal lymph node marks the onset of metastasis (5). The general sites of metastases are brain, liver, bone and adrenal glands.

1.2 Treatment Options

Classical therapeutic options available for the treatment of malignant neoplasms are surgery, chemotherapy and radiation therapy. Treatment of lung cancer depends on the stage and type of the disease, when first diagnosed. If the tumor is confirmed as one of the NSCLCs and is localized to a specific portion of the lung, then surgical resection (removal of the primary tumor) is the most effective form of treatment. Adjuvant chemotherapy after surgery is found to be beneficial to the patients when compared to patients who undergo surgery alone (6). If the tumor has the properties of SCLC and is diagnosed at a highly metastatic stage, then radiation therapy and chemotherapy are the most effective forms of treatment to eradicate widespread disease. Chemotherapy is generally preferred in almost all stages of lung cancer either as mainstream therapy or as
adjuvant therapy. Depending on their target sites, the chemotherapeutic agents are currently classified as follows:

a) **Anti–angiogenic agents**: they prevent the formation of new capillaries and blood vessels for the nourishment of the tumor (7).

b) **Vascular disrupting agents**: the two main types of vascular targeting agents (VTAs) are currently being developed for cancer treatment. The ligand directed VTAs which use antibodies and peptides to target specific toxins on the tumor endothelial cells, and small molecule antitubulin agents which have inherent VTA activity. The antitubulin agents exploit differences between the tumor and normal endothelial cells and cause selective occlusion of the vascular blood supply, limiting nutrients to the developing tumor (8, 9).

c) **Drugs targeting tumor cells**: Conventional chemotherapeutics are formulated to target tumor cells within the interstitial matrix. However, this approach is mostly influenced by physiological features of the tumor and its micro environment (11).

### 1.3 Limitations of conventional chemotherapy

Toxicity to the normal tissues of the body and development of cellular resistance to antineoplastic agents have been the principle obstacles limiting the effects of conventional chemotherapy. The physiological and biochemical differences in the tumor microenvironment also act as formidable barriers to blood borne chemotherapeutic drug molecules. The characteristics of tumor vasculature are as follows:

**Heterogenous blood supply**: Tumor blood vessels often have large diameters which may influence vascular permeability of circulating macromolecules. The distribution of
vessels is also heterogeneous. Hence, the tumor microenvironment is physically differentiated into a necrotic central region and a peripheral rim of rapidly proliferating cells. This heterogeneity leads to uneven distribution of chemotherapeutics in solid tumors (10).

The Tumor interstitial matrix- The characteristics of the tumor interstitium hinder the transport of drug molecules through the large interstitial space. These characteristics include high collagen, high proteoglycan and hyaluronate content, elevated interstitial fluid pressure, absence of anatomically well-defined functional lymphatic networks. All of these factors influence interstitial diffusion and transport of macromolecules (11, 12).

Our existing knowledge of the physiology of solid tumors as well as the molecular pathways underlying cytotoxic effects of the chemotherapeutic agents have both provided useful information for developing innovative therapeutic strategies. One such strategy is to minimize the nonspecific accumulation of drugs in normal tissues of the body by delivering the drugs to the tumor vasculature. The goal of any vascular disrupting agent is to migrate exclusively to the tumor vasculature and shut down oxygen and nutrient transport to tumor cells. To achieve this, we must encapsulate the drug in a macromolecular carrier system, such as liposomes, decrease the amount of non-specific accumulation, and increase the amount of drug at the tumor vascular site.
1.4 Chemotherapeutics targeting Microtubules

There are different classes of chemotherapeutic agents depending on chemical specificities, biochemical pathways and specific mechanisms by which drugs exert their cytotoxic effects on cellular targets. The microtubule targeting agents are one of the extensively studied classes of drugs, which are capable of targeting the pre-existing vascular supply and shutting down blood flow to solid tumors. This specific class consists of many small drug molecules derived from natural and synthetic sources (13, 14). Many potent microtubule disrupting agents cannot be used clinically because of a delicate risk to benefit ratio. One such natural alkaloid is colchicine, extracted from the stem of saffron meadow (*Colchicum autumnale*). Colchicine is the drug of choice for treatment of gouty arthritis and Familial Mediterranean fever (15, 16).

The colchicine binding site on tubulin protein is well characterized. In fact, tubulin was first discovered as a tubulin-colchicine complex and was later isolated as pure tubulin (17). Microtubules, the structures that form the cytoskeleton, are made up of tubulin heterodimers. They are involved in various cellular processes such as cell division, maintenance of cell shape, cell signaling, cell migration and cellular transport. Colchicine binds at the interphase of α and β subunits of the tubulin heterodimer (18). Colchicine induced conformational changes in the tubulin heterodimer interferes with the growth of microtubules by destabilizing them. The dynamic instability of the microtubule is destroyed because of the conformational changes in the amphipathic helix at the carboxy terminal of beta tubulin, which serves as a site for GTP hydrolysis or GDP-Pi capping (19). A poorly reversible tubulin-colchicine (TC) complex is formed due to high activation energy and favorable entropy of the complex (20). The TC complex does not
prevent the addition of tubulin to the growing end of the microtubule, but decreases the rate of addition of heterodimers to the microtubule because this addition is energetically unfavorable, and eventually leads to disruption of microtubules (16).

The destruction of microtubules subsequently disrupts the cytoskeleton of the cell and thus interferes with all the microtubule associated biochemical functions indicated above. The disruption of microtubules first induces an increase in the focal adhesion points and actin contractility and then produces a sudden decrease in cell adhesion profile due to disorganization in focal adhesion points and actin stress fibers (20). The series of events result in cell rounding, cellular detachment from the extracellular matrix and eventually cell death (20). Colchicine is also known for its classic antimitotic activity, as the microtubules are indispensable during a mitotic cell division. It is a phase specific drug acting on G1-phase of the cell cycle, preventing the formation of proper mitotic spindle and disrupting the microtubules, thus preventing mitosis (19). The endothelial cells lining the microvasculature also undergo cytoskeletal damage and this brings about tumor ischemia and necrosis, alterations in functions of endothelial cells, change in cell structure and in vascular blood flow dynamics resulting in complete vascular shutdown in solid tumors (21). At this juncture, one has to deal with the fact that the starvation of the tumor resulting in tumor death may point to many possible intrinsic mechanisms, which might respond to colchicine treatment. For instance, interaction of circulating leukocytes with the tumor microenvironment is essential for the induced recruitment of angiogenic effectors towards tumor vessels (22). This induction up regulates the production of cytokines like TNF-α and IL-α leading to activation of endothelial cells. The adhesion molecules like selectins are overexpressed by the activated endothelial cells (23). E-
selectin is localized on endothelial cell venules and capillaries and aids in the inflammatory process by enabling the initial attachment of inflammatory cells like neutrophils and leukocytes to the endothelium. An increase in e-selectin production on endothelial cells maximizes cell adhesiveness, leukocyte rolling and transmigration into angiogenic blood vessels (22). The tetrasaccharides like sialyl lewis\(^x\) and sialyl lewis\(^a\) act as ligands for the e-selectin and other homologous selectins (L,P-selectins) in general (23, 24). In addition, e-selectin on endothelial cells is thought to interact with cancer cells which have aberrant expression of oligosaccharides like sialyl lewis\(^x\) and sialyl lewis\(^a\) and play a very important role in tumor progression for two reasons (25). First, tumor metastasis is facilitated by attachment of the ligands to the e-selectin receptors on endothelial cells, and second, excessive presence of the oligosaccharide is rejected by natural killer cells impairing the immune response (25). Colchicine affects the display of e-selectin qualitatively but does not induce any significant quantitative changes. The dispersion of e-selectin away from the site of expression results in the delay in transmigration of leukocytes and tumor cells decreasing the inflammatory response and the metastasis of tumor cells respectively (26), and thus functions as an antiangiogenic agent. All the above mechanisms clearly stress the potential of vascular targeting tumor therapy by tubulin-binding agents exerting significant antivascular effects \textit{in vivo}, as has been reported for other vascular disrupting agents such as combretastatin A-4 (27, 28) and ZD6126 (29) and other colchicine analogues.
1.5 Colchicine Toxicity

Although colchicine is a promising drug candidate, targeting both tumor vasculature and angiogenesis, the concentrations at which it elicits these effects always approach the maximum tolerated dose (MTD) (30). Colchicine was actually used to disrupt both animal and human tumors as early as 1930’s, but the drug never reached the clinical settings because of serious toxic side effects (31-34). Colchicine, being a very toxic drug, cannot be prescribed at high doses for oral delivery (35-38). In cases of acute attack, intravenous administration is restricted to a dose of 4-5 mg (2 -3 mg/dose). The intravenously administered dose is usually ½ the dose administered orally (38). Absolute therapeutic window should be followed during IV administration, since fatal mishaps have been reported when drug was used at higher concentrations (39, 40). The nonspecific accumulation of colchicine has resulted in severe gastrointestinal disturbances leading to neuromuscular toxicities (41) and multisystem failure (42). Common side effects include alopecia, leucopenia, nerve irritancy and teratogenic effects in mice etc (35-37). Colchicine injectables dispensed by the licensed pharmacists are often recalled by the FDA due to unpredictable deaths associated with the potent nature of the drug (43).

1.6 Development of a delivery Vehicle

In order to mask the toxicities of the drug and take full advantage of therapeutic benefits, colchicine should be targeted specifically to tumors. Such an approach would significantly minimize damage to normal tissues. As the tumor vasculature is known to harbor many distinctly different physiological and biochemical features from the normal
vasculature, many of these differences can be exploited by novel drug carrier molecules for treatment (27, 30). For this reason, the development of a delivery vehicle that is highly selective to the tumor vasculature and can release the drug only when it reaches the site of action is an ideal way to minimize the toxicities associated with the use of colchicine alone.

1.7 Liposomes as drug delivery vehicles

The physiological barriers in the microenvironment of the tumor mass obstruct the effective delivery of drugs to specific targets (12). Liposomes have revolutionized targeted drug delivery, especially to solid tumors. Liposomes possess specific physicochemical properties which can be harnessed to overcome the above barriers and optimize targeted drug delivery (44-46). A conventional liposome is a macromolecular carrier composed of a lipid bilayer enclosing an aqueous core. They enable the safe and efficient loading of a hydrophilic drug into the aqueous core and predominantly hydrophobic drugs within the lipid bilayer. The liposomal drug when injected through IV route also has a lower volume of distribution and the concentration of the drug is increased at the target site (47). The lower volume of distribution results in a decrease in nonspecific drug accumulation and a significant reduction in drug associated side effects. Since liposomes are composed of natural biological lipids, they are biocompatible, biodegradable and non-toxic when used at optimal doses. Liposomes also protect drugs from the harsh environments in the circulation and prevent the early metabolism and inactivation by hepatic enzymes. The selectivity of liposomal accumulation is based on size limitations. They cannot be extravasated across the healthy vasculature, but when
they reach the leaky tumor microvasculature, fenestrations in the endothelial lining enables the transport into tumor tissues, where the chemotherapeutic agent is released. All of these properties may contribute to a significant tumor response to chemotherapy compared to the free drug itself.

1.8 PEG modified Cationic Liposomes (PCLs).

Although conventional liposomes seem to be the ideal carriers of chemotherapeutic agents, in practice they are rapidly cleared from circulation by the first line of defense, the macrophages of the reticuloendothelial systems (RES) including the liver and spleen (48). The serum opsonins are thought to play a critical role in recognition and subsequent clearance by RES macrophages (49). Therefore, drug delivery to tissues other than the RES will require further modification, which would limit their uptake by macrophages. This can be achieved either by directly preventing the interaction of liposomes with the receptors on macrophages, or indirectly, by decreasing the binding of serum opsonins. The long circulating liposomes were thus formulated with polyethylene-glycol (PEG) (50). PEG is a flexible chained hydrophilic polymer which occupies the periliposomal layer and tends to oust the antigenic molecules from the liposome membrane surface (49, 51-54). Consequently, the binding of blood serum opsonins to the surface of liposomes is minimized which decreases uptake by RES macrophages (47, 48, 50, 53). PEG also minimizes aggregation of liposomes in solution, as the steric hindrance induces repulsive forces between the liposomal surfaces (54).

Liposomes bearing a cationic surface charge deliver the chemotherapeutic agents preferentially to endothelial cells of the tumor vasculature, leading to disruption of the
vascular blood supply to solid tumors (27, 29). Liposome preparations containing 50 mol% DOTAP content (a cationic lipid) renders sufficient positive charge to liposomes and is responsible for the preferential accumulation of liposomes along the tumor vasculature (44). The accumulation of liposomes with 50% DOTAP was significantly higher in the lungs compared to preparations containing < 50% DOTAP content (44). The DOTAP and other cationic lipids significantly improve the incorporation efficiency of drugs such as paclitaxel, 5FU, doxorubicin, cisplatin and etoposide in liposomes (46, 55).

**Fig-1:** Colchicine loaded PEGylated cationic liposomes – 50 % DOTAP renders a positive charge to the preparation. Colchicine being an amphiphilic drug might be distributed both within the aqueous core and in the bilayer compartment.
1.9 PEGylated Cationic Liposomes targeting Tumor Vasculature.

The vascular supply of solid tumors is heavily dependent on the tumor microenvironment. The microenvironment also influences the rate of tumor growth and metastasis. Many strategies have evolved to target chemotherapeutic drugs specifically to tumors; however, targeting tumor endothelial cells has gained tremendous support. The tumor vasculature, as in the normal vasculature, is lined with endothelial cells. The endothelial cells do offer some advantages over the other therapeutic targets for development of a successful anticancer therapy. Some examples are as follows. The proliferation rate of tumor endothelial cells is 50–1000-fold higher compared with the proliferation rates of normal endothelial cells (56, 57). Endothelial cells are mostly resistant to mutations, which minimize the problem of drug resistance. Furthermore, any kind of solid tumor can be targeted with this strategy irrespective of the origin of the tumor. Most importantly, endothelial cells are uniquely exposed to blood borne chemotherapeutic agents, which solves the problem with drug delivery to deep regions within solid tumors, because it is not necessary to target this region of tumor in order to achieve therapeutic success. Angiogenic endothelial cells take up cationic liposomes by receptor-mediated endocytosis, charge-dependent binding and by a potentially altered glycocalyx of tumor microvessels where expression of surface molecules is 15-33 fold higher than in the normal endothelium (58). The significant negative surface charge density supports the development of charged xenobiotics and drug delivery vehicles for tumor vascular targeting. Although PCLs mainly target tumor vasculature, some accumulation has also been observed in the tumor in interstitial matrix (43). Given the significant levels accumulated, the tumor vascular compartment is the primary target of
colchicine loaded experimental therapeutics as shown in fig-2. Thus, we aim to develop colchicine-loaded-PEG modified cationic liposomes using DOTAP as the cationic lipid of choice for treatment of Lewis Lung Carcinoma, using in vitro models of human cancer disease (Fig-2).

![Diagram](image)

**Normal Tissue**

- Endothelial Cells
- PCL-Colchicine
- Release of Colchicine

**Tumor Tissue**

- Tumor Cell
- Endosome
- Endothelial Cell

Fig 2: Delivery of Colchicine loaded Pegylated Cationic Liposomes (PCLs) and preferential accumulation in tumor tissue when compared to normal tissue. Receptor mediated endocytosis into tumor cells and endothelial cells are shown. The electrostatic interaction between the tumor endothelial cells and PCLs also aid in selective uptake. A significantly reduced fraction of injected dose compared to the tumor vessels will accumulate in the tumor interstitial matrix as well.
2. Statement of Hypothesis

We hypothesize that

- Colchicine can be loaded in PCLs with high incorporation efficiency
- When colchicine is loaded in PCLs we will observe more significant drug effects compared to the effects of colchicine alone.
- The effect of colchicine on microtubules can be studied using fluorescence microscopy.
- We will observe benefits of using colchicine-PCLs against activated endothelial cells \textit{in vitro} compared to the effects of colchicine alone.

3. Specific aims

Aim 1 - To develop and characterize colchicine-loaded PCLs for tumor vascular targeting

The particle size, zeta potential of PCLs were determined using particle size analyzer; percent incorporation of colchicine into PCLs was determined by reverse phase HPLC.

Aim 2 – To determine cell growth inhibitory properties of colchicine and liposomal colchicine.

The growth inhibitory effects of free drug and liposomal drug were evaluated against Lewis lung Carcinoma (LLC) and pancreatic endothelial cells (MS1 VEGF).

Aim 3 – Qualitative analysis of the effects of liposomal colchicine on cytoskeleton and nuclear areas using Bioquant image analysis.

The extent of cytoskeletal damage is quantified after free drug and liposomal drug treatment in MS1 VEGF and LLC and compared with untreated controls. Experiments
will be carried out using fluorescence microscopy performed on cells grown in 6 well plates.

**Aim 4 – To determine the effect of liposomal colchicine on the activated endothelial cells using *in vitro* model.**

### 4. Materials and Methods.

#### 4.1 Materials

Colchicine, monoclonal Anti β tubulin FITC conjugate and purified mouse immunoglobulin (F 2043) were purchased from Sigma Aldrich (St.Louis, MO). Slow Fade® Gold antifade reagent with DAPI was purchased from Molecular Probes (Invitrogen Life Technologies, Carlsbad, California). Ammonium Acetate and Bovine Serum Albumin (BSA) were purchased from Sigma Aldrich (St.Louis, MO). Chloroform, 1% Acetic acid, absolute methanol and acetone were purchased from Fisher Scientific (Pittsburgh, PA). DOPC, DOTAP, Cholesterol, PEG-5000 were purchased from Avanti Polar Lipids (Alabaster, AL). Lewis Lung Carcinoma (LLC), Human Pancreatic Endothelial cell line (MS1-VEGF), maintenance media Dulbecco’s Modification of Eagle’s Medium (DMEM) and Fetal Bovine Serum (FBS) were purchased from ATCC (American Type Culture Collection, Manassas, VA). Phosphate buffered saline (PBS) 1X was purchased from Cambrex, NJ.

#### 4.2 Preparation of free drug Solution

An amount of 10 µmole/ml of colchicine stock solution was initially prepared in methanol. Methanol was evaporated using a rotary evaporator (J-KEM Scientific, Inc.,
St. Louis, MO). Aliquots were freshly prepared with a 1:10 dilution in sterile 1X PBS buffer for most studies involving use of free drug unless otherwise noted.

4.3 Preparation of Liposomes

All the lipids involved were initially stored as solutions in chloroform at 10µmol/ml concentrations and the liposomes were prepared using a thin film evaporation technique (45, 59). Colchicine and lipid stock solutions were prepared at 10µmole/ml. The colchicine loaded pegylated cationic liposomes were prepared at 3, 5 and 10mole% concentrations. Cationic charge was contributed by 50% DOTAP. A lipid mixture was prepared with DOPC:DOTAP:Cholesterol:DOPE-PEG-5000 (35:50:10:5) and evaporated to dryness at 42°C with the aid of a rotary evaporator to obtain a thin film of lipid. Any residual amount of organic solvent was removed by subjecting the lipid film to freeze drying for an additional 2 hrs using Labconco freeze dryer (Labconco Corporation, Kansas City, MO). The film was then hydrated with 1X PBS to form multilamellar liposomes. Liposomes were vortexed intermittently and put in a water bath set at 44°C and in a refrigerator at 4°C for 30-60 min increments. The multilamellar liposomes were sonicated in a bath type sonicator (Laboratory Supplies Corporation, Hicksville, NY) to yield homogenously distributed unilamellar liposomes. Liposome sizes and zeta potentials for colchicine loaded PEGylated cationic liposomes were measured at 25°C in double distilled water using the 90 PLUS particle size analyzer (Brookhaven Instruments, Holtsville, NY).
4.4 Incorporation Efficiency Studies - UV Spectroscopic analysis

The incorporation efficiency of colchicine into liposomes at various concentrations was determined by using the UV Spectrophotometer, μQuant (Bio-Tek Instruments Inc., Winooski, VT). The results were confirmed using HPLC-Waters (Milford-MA). The protocol followed for the preparation of mobile phase and running the sample has been reported elsewhere (60). The analytical Column, Discovery SUPELCO® (Sigma Aldrich, St.Louis, MO) was operated at ambient temperatures. Isocratic elution with A: 48% CH$_3$COONH$_4$ 0.05M; and B: 52% CH$_3$OH was used at the flow rate of 1.0mL/min. The colchicine loaded liposome preparation was centrifuged at 15,000 rpm (IEC-Micromax RF, Waltham, MA) to separate unbound drug and any lipid debris from the liposomal drug. Dialysis was carried out for 12 hrs in PBS buffer (pH 7.4) using Float-A-Lyzer®, MW=500 (Spectrum Laboratories, Inc., Rancho Dominguez, CA). UV absorbance of colchicine was measured at 245nm (60). The liposomal preparations (before and after separation) were ruptured with methanol and samples were analyzed for the amount of colchicine released in each case. The percent drug incorporated was calculated as follows.

\[
\text{Percent Drug Incorporated} = \frac{\text{UV absorbance for liposomes after centrifugation/Dialysis}}{\text{UV absorbance for liposomes before centrifugation}} \times 100
\]

Each bar in the graph represents Mean ± SDs of three different experiments under identical conditions.

4.5 Cytotoxicity Studies

The percent cell viability was determined by sulforhodamine B assay (45, 46). All percent cell viability studies were carried out in a 48 well plate (1×10$^4$ cells/ml).
purchased from Fisher Scientific (Pittsburgh, PA). Cells were exposed to drug, liposomal drug or liposomes only for 24 hrs. Wells were washed with PBS to remove unbound liposomes and cellular debris and the cells were fixed to the plate by adding 100 µl of 50% TCA into each well followed by incubation at 4 °C. After 60 min TCA was washed off cells and plate was incubated with 200 µl of 0.4% sulforhodamine B for 30 min in dark. The dye was then washed off with 1% acetic acid and air-dried in a laminar flow hood. Fluorescence intensity (FI) was measured by dissolving bound sulforhodamine with equal volumes of 1× PBS in all wells, at an excitation wavelength of 550 nm, and emission wavelength of 590 nm using a FLX 800 Fluorescence Microplate Reader, Bio-Tek Instruments Inc., (Winooski, Vermont). Percent of cell viability was calculated by the following formula:

\[
\text{Percent of cell viability} = \frac{F.I \text{ of treated cell population}}{F.I \text{ of untreated cell population}} \times 100
\]

The percent of cell viability was determined and plotted as a function of concentration of drug (fig-4).

4.6 Fluorescence Microscopy

Fluorescence microscopic analyses were carried out using a BX61 W1 Olympus fluorescence microscope from Optical Analysis Corporation (Melville, NY). Cells were seeded at 5*10^5 onto sterile coverslips in a 6 well plate (Corning, NY). After 24 hrs of incubation at 37°C, rhodamine labeled liposomes (cell association), free drug or liposomal drug was added to selected wells. The well plate was incubated at 37°C for
another 24 hrs after which the coverslips were prepared for mounting. The procedure followed for cell fixation and staining with antibody is according to the instructions provided in the data sheet of the FITC-conjugated β tubulin antibody. The coverslips were washed twice with 1X PBS to remove cellular debris and then fixed with cold absolute methanol. Coverslips were then incubated at -20°C for 10 min. The methanol was washed off with cold acetone and the cells were rehydrated in 1X PBS for at least 30 min. The rehydrated cells were then incubated with Fluorescence Isothiocyanate (FITC) conjugated β-tubulin antibody for 1 hr, suitably diluted with 1X PBS made up with 1% BSA. A working dilution of 1:25 was used. Excess antibody was washed off with 1X PBS at least three times, and samples were then mounted onto a glass slide using Slowfade® Gold antifade reagent DAPI as the mounting media. The images were randomly selected at five different fields on the coverslip. DIC (Differential Interference Contrast) microscopy was used to observe cell shape and morphology prior to obtaining all fluorescence images. The FITC and DAPI images were finally merged to observe the area of cytoskeleton and nucleus using LLC and MS1-VEGF cell lines. For cell association studies (Fig 12) the rhodamine labeled liposomes were visualized using rhodamine filter and merged with the FITC and DAPI images. All images were captured under 40X objective using a BX61 W1 Olympus Fluorescence microscope from Optical Analysis Corporation (Melville, NY).
4.7 Bioquant – Quantitative analysis

BIOQUANT (Image analysis Co-operation) was used to quantify the merged images of the cell nucleus and cytoskeleton. Images were selected randomly at five different fields on the coverslip. Areas with similar fluorescence intensities of FITC (cytoskeleton) and DAPI (nucleus) were manually selected and measured (Fig-3). The total number of cells in each field was counted, and the area occupied by one cell was calculated as follows.

\[
\text{Cytoskeleton area per cell} = \frac{\text{Total Cytoskeletal area measured}}{\text{Number of cells in the field}}
\]

\[
\text{Nuclear area per cell} = \frac{\text{Total nuclear area}}{\text{Number of cells in the field}}
\]

Mean cytoskeleton area and nuclear area per cell was plotted in a bar graph. Each bar in the graph represents Mean ± SD of five different experiments. Statistics were performed using ANOVA (SPSS 15) at a significance level of 0.05.

4.8 Cell Association

All Cell association studies for LLC and MS1-VEGF were carried out in 24 well plates (2 x 10^4 cells/ml) purchased from Fisher Scientific (Pittsburg, PA). Rhodamine labeled liposomes were prepared by incorporating 5 mole % rhodamine into the lipid mixture and following the method described above. The cells were incubated with required cell density at 37°C and 5 % CO₂ overnight to allow for cell attachment. The rhodamine labeled liposomes were added at 50 nmol/mL concentration into each well and incubated for optimum association. The association was measured at 4, 24 and 48 hrs for both the cell lines after adding the liposomes. The wells were washed twice with 1x PBS to
remove the debris and 1mL of 1xPBS was added to wells. The fluorescence intensity of rhodamine labeled liposomes taken up by the cells was measured using a fluorescence microplate reader. The Fluorescence intensity-arbitrary units were plotted against time (hrs) and each bar represents the mean ± SD of two different experiments. (Fig 11).

4.9 Statistical analysis

Unless specified otherwise, the statistical analyses of all studies was carried out using ANOVA at $\alpha = 0.05$. SPSS 15 is software used. * $p< 0.05$, ** $p< 0.001$. 
Fig-3: BIOQUANT Analysis: The figure above shows the procedure for quantifying the fluorescence images of MS1-VEGF. The green fluorescence was acquired using a FITC filter and represents the cytoskeleton of the cell and blue represents the nucleus. Verification of total cytoskeleton and nuclear areas were determined by measuring the amount of green and blue respectively and dividing by the total number of cells in the field. The result was mean cytoskeleton and nuclear areas per number of cells in the field.
Results

Fig-5: DIC images of LLC and MS1-VEGF, *in vitro* models

Fig-4: Determination of the standard curve for working concentrations of colchicine (n mole/ml) (Fig A). Concentration of colchicine (mole %) is plotted against % of drug incorporated in PCLs. (Fig B) UC-uncentrifuged sample; C-Centrifuged sample; C+D-sample subjected to both centrifugation and dialysis. Maximum drug incorporated was observed at 5 mole % after which there was no significant increase when compared to 10 mole %. * p < 0.05, ANOVA.
Fig 6: The % of cell viability of LLC and MS1-VEGF against increasing concentrations of colchicine was determined by SRB assay and use of a florescence microplate reader. A stabilizing drug effect was observed around 10nmoles for MS1-VEGF. In general, LLC cells were more susceptible to effects of colchicine, but no lower than 60 % viability was observed for either cell line.
**Fig-14:** The graphs above represent the dose response curve of LLC (A) and MS1-VEGF (B) cell lines. Colchicine is a microtubule destabilizing agent whereas Taxol is a microtubule stabilizing agent.
Fig -7: Cytotoxicity: Percent of viability of LLC and MS1 VEGF cells. UT- Untreated, FD- colchicine alone; LD-colchicine-loaded PCLs. At all concentrations evaluated colchicine PCLs show a significant effect on cell viability when compared to the effect of free drug.* p < 0.05, ANOVA.
Fig-8: The fluorescence images above show the disruption of microtubules with colchicine alone and colchicine loaded in PEGylated cationic liposomes. The cells were fixed with methanol before incubation with FITC conjugated β-tubulin antibody for 1 hr. FITC and DAPI images were merged using images captured at 40X magnification. FITC stained cytoskeleton (in green) and DAPI stained the nucleus (in Blue). LLC (A-F). (A) – Untreated Controls, (B)- Liposomal Controls, (C)- colchicine treated, (D)- Liposomal Drug treated, (E) – cytoskeleton area per cell, (F) – nuclear area per cell. Each bar in the graphs represents Mean ± SD of five different experiments. * p < 0.05; ** p < 0.001.
Fig-9: The fluorescence images above show the disruption of microtubules with colchicine alone and colchicine loaded in PEGylated cationic liposomes. The cells were fixed with methanol before incubation with FITC conjugated β-tubulin antibody for 1 hr. FITC and DAPI images were merged using images captured at 40X magnification. FITC stained cytoskeleton (in green) and DAPI stained the nucleus (in Blue). MS1-VEGF (G-L). (G)–Untreated Controls, (H)- Liposomal Controls, (I)- colchicine treated, (J)- Liposomal Drug treated, (K) – cytoskeleton area per cell, (L) – nuclear area per cell. Each bar in the graphs represents Mean ± SD of five different experiments. * p < 0.05; ** < 0.001.
Fig-10: The DIC images were acquired using a Fluorescence microscope at 40X objective. Images show morphological changes of MS1-VEGF and LLC following treatment with colchicine.
Fig -11: Time-dependent cellular uptake of rhodamine labeled liposomes: At all time points evaluated PCLs show significantly higher association with MS1-VEGF when compared to LLC cells. There was no significant increase in the uptake of liposomes observed following 24 hrs of incubation with either cell lines.

* p < 0.05, ANOVA
Fig-12: The fluorescence images above show the uptake of rhodamine labeled PCLs (red) with respect to the cytoskeleton (green) and nuclear (blue) areas. All the images were captured at 40X magnification using a fluorescence microscope. It is evident from the above images that a lesser amount of PCLs were taken up by LLC (A) compared to MS1-VEGF (B) cells.
**Fig-13:** MS1-VEGF Vs LLC: The above picture shows the cytoskeleton area, cellular uptake of liposomes and cell size of MS1-VEGF cells in comparison to LLC cells. MS1-VEGF cells have a significantly higher cytoskeleton surface area and cell size that may have contributed to the significant uptake of PCLs by these cells.
<table>
<thead>
<tr>
<th></th>
<th>Particle Size (nm)</th>
<th>Zetapotential $\zeta$ (mv)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electroneutral Liposomes (ENLs) [DOPC+ Cholesterol + PEG + Colchicine]</td>
<td>133.36 ± 11.61</td>
<td>-30.18 ± 4.31</td>
</tr>
<tr>
<td>Cationic Liposomes (PCLs) [DOPC+ DOTAP+ Cholesterol + PEG + Colchicine]</td>
<td>126.53 ± 2.54</td>
<td>22.35 ± 7.12*</td>
</tr>
</tbody>
</table>

Table-1: The Particle Size and Zeta potential of the final formulation are reported here. The ENLs are colchicine-loaded liposomes without DOTAP. PCLs are colchicine-loaded PEGylated cationic liposomes with 50 % DOTAP. All of the formulations contain 5 mole% colchicine, they were subsequently used in the fluorescence microscopy studies for further analysis.

* p < 0.05, Student t test compared to ENLs.

5. Results

The research involving the use of liposomes as a drug delivery vehicle especially for the delivery of chemotherapeutic agents has reached its zenith after Doxil®. This success story opened a huge arena for reconsidering the use of many potent therapeutic agents that have lost their importance in the pages of history, as they are highly toxic. Colchicine is one such example used today as a lead compound to compare with other antimitotic drugs. We therefore propose to develop a ‘magic bullet’ with colchicine by incorporating it into PEGylated cationic liposomes. Our goal is to target colchicine PCLs to the tumor vasculature and hence formulate the vehicles with an optimum positive charge to promote the electrostatic interactions with endothelial cells of tumor vasculature.
5.1 Physicochemical Characteristics of Colchicine PCLs

The physicochemical characteristics of liposomes play an important role in defining the targeting properties of the final formulation. We formulated PCLs with 3, 5 and 10 mole % colchicine. To ensure a homogenous liposomal preparation, liposomes were sonicated for 10 min and the particle size and zeta potential were determined. We report the values for 5 mole % colchicine formulations since this particular drug to lipid ratio was used for our fluorescence microscopy studies. We initially formulated electroneutral liposomes (ENLs), without using DOTAP, a synthetic cationic lipid, and the particle size was determined to be 133.36 ± 11.61 nm. The inclusion of 50% DOTAP in drug loaded PCLs resulted in a significant increase in zeta potential of, 22.35 ± 7.12 mv when compared to the zeta potential of ENLs which was -30.18 ± 4.31 mv. Although there was a decrease in the particle size, 126.53 ± 2.54 nm, it was not statistically significant when compared to ENLs. (Table-1).

5.2 Drug Incorporation Studies

Investigations into the structure of liposomes have revealed that drug molecules can incorporate in either the lipid bilayer or the aqueous core. Colchicine, being an amphiphatic molecule can occupy the bilayer as well as the aqueous core. Initially we determined a working curve of absorbance versus known concentrations of colchicine (10 – 100 nmole/ml). The standard curve \( r^2 = 0.9956 \) reflects a linear relationship between the absorbance and the concentration of colchicine, suggesting that the system follows the Beer-Lambert’s law. The formulations were centrifuged and dialyzed to separate the
free drug from drug incorporated in liposomes. We determined the incorporation efficiency of colchicine at 3, 5 and 10 mole % concentration by HPLC analysis. The AUC under the peaks in each case was used to determine the concentration of colchicine in the sample. The percent of drug incorporated was calculated as described above and depicted in the bar graph. The percent of colchicine incorporated into PCLs at 3, 5, 10 mole% after centrifugation was 89.30 ± 0.64, 99.80 ± 7.6 and 100.31 ± 11.11 whereas in dialyzed varieties the percent incorporation is 34.96 ± 16.36, 67.85 ± 7.02, 73.96 ± 7.74 respectively. Generally, there was no significant decrease observed in the percent of colchicine incorporated in the centrifuged samples but incorporation was significantly lowered in the dialyzed variety compared to the uncentrifuged preparations. We also observed a linear increase in the incorporation with increase in mole percent of colchicine up to 5 mole%, although there was no significant change observed in the dialyzed variety at concentration as high as in 10 mole% formulation.

5.3 Cell Viability Studies

We determined the effect of colchicine and liposomal colchicine (PCLs) on LLC and MS1-VEGF using SRB assay. Sulforhodamine is a protein dye which binds electrostatically to basic amino acid residues of trichloroacetic acid-fixed cells. Under mild acidic conditions it can be extracted from cells and solubulized for measurement using a Fluorescence microplate reader. We report the percent cell viability after treatment with free drug and liposomal drug as a function of increasing concentration of colchicine (5-500 n mole/ml). We observed a stabilizing drug effect around 10 n moles/ml for MS1-VEGF where the percent cell viability was 82.35 ± 8 and did not show any
significant increase in cytotoxic drug activity at 500 nmole/ml. In general, LLC cells were more susceptible to the effects of colchicine, although the stabilizing effect was observed at all concentrations below 100 nmole/ml. Above this concentration there was a significant decrease in the percent of cell viability between 200-500 nmole/ml. No lower than 60% cell viability was observed in either cell line (fig-6). Percent cell viability with liposomal colchicine was determined at 5 mole% concentration and compared to free drug effect at the equivalent concentrations. At 50 nmole/ml the percent cell viability of LLC with liposomal colchicine (46.6 ± 7.8) was significantly lower than the free drug (71.82 ± 2.6) (fig-7).

5.4 Fluorescence microscopy

Fluorescence microscopic analysis was carried out using both cell lines to evaluate the disruption of microtubules with free drug and liposomal drug treatment. The FITC conjugated β tubulin antibody in green and DAPI in blue is reflective of the microtubule protein and nucleus respectively. The mean cytoskeleton area/cell in LLC cells was significantly lower after both free drug (1841.3 ± 725.8) and liposomal drug (528.24 ± 41.10) treatment when compared to untreated controls (4554 ± 748.5). The nuclear area did not show a significant change with the free drug (1562 ± 588) treatment although liposomal drug (449.8 ± 50.25) had a significant effect on the nucleus when compared to the untreated controls (2016 ± 351.7). The endothelial cell line MS1-VEGF was more sensitive to the effects of liposomes and hence the lipids were used at 500 nmole/ml, this was 1/2 the concentration used for the LLC. The mean cytoskeleton area/cell in MS1-VEGF cells was significantly lowered after treatment with both free drug (4305 ± 485.5)
and liposomal drug (2241 ± 145) treatment when compared to untreated controls (10645 ± 1199). The liposomal controls also show a significant decrease in the cytoskeleton area, but had no significant effect on the nuclear area in this case. The nuclear area revealed a significant change with the free drug (1875 ± 34) treatment although liposomal drug (1539 ± 55) had significant effect on the nuclear area when compared to untreated controls (2288 ± 360). Interestingly, the cytoskeleton area of MS1-VEGF cells (10645 ± 1199) was two fold higher than that of the LLC cells (4554 ± 748.5) in the untreated control group, with no significant change in nuclear area.

5.5 Cell Association Studies

The cell association studies with LLC and MS1-VEGF were performed using rhodamine labeled liposomes (500 nmole/ml) at 0.5, 4, 24 and 48 hrs. From Fig-12 the MS1-VEGF exhibits significantly higher association with liposomes at 24 hrs (71413 ± 2665) when compared to LLC (47051 ± 2962) at the same time point. A significant increase in cellular uptake from 0.5 hrs to 4 hrs and from 4 hrs to 24 hrs was observed in both the cell lines. There was no significant increase observed in the cell association at 48 hrs with LLC (56089 ± 4933) or MS1-VEGF (77627 ± 6938.6). These results indicated that a significantly higher number of PCLs were taken up by the endothelial cell line when compared to the lung cancer cells. This study also supports the use of the 24 hr time point and these cell lines to conduct other assays involving the cellular uptake of liposomes.
6. Discussion

After two decades of constant effort to attenuate the toxicities associated with the use of conventional chemotherapeutic drugs, the formulation of pegylated cationic liposomes (PCLs) as drug delivery vehicles introduced a new trend in the development of targeted drug delivery to the tumor vasculature (44, 46, 61). This approach certainly has an advantage over the use of other conventional chemotherapeutic approaches. Firstly, a single vessel provides nutrition and eliminates waste products from thousands of tumor cells, and hence, damage caused to a few endothelial cells can influence the flow of blood upstream or downstream (9). Secondly, it is not necessary to kill an endothelial cell to produce a desired effect, changing of the shape and morphology of the cell might be enough to occlude or completely shut down vascular function in that particular tumor region (9) thus supporting the rationale for choosing colchicine as the drug of choice for the proposed studies.

Here we assume, and discuss, the mechanistic role of PCL-colchicine in the treatment of human endothelial and lung cancer cells. The free drug profile of colchicine showed a stabilizing effect on MS1 VEGF, rather than a typical dose response curve as in the case of taxol (Fig-14). The stabilizing effect can be attributed to the disruption of spindle microtubules and blockage of cell division. This results in cell cycle arrest which prevents cellular proliferation at relatively low concentrations when compared to other vascular disrupting agents (18, 26). Hence, it can be assumed that the cell line with the fastest turnover will be the most susceptible to the effects of treatment. This is consistent with our observations where LLC cells are significantly more susceptible to the effects of colchicine compared to MS1 VEGF cell line. There is considerable evidence suggesting
that tumor endothelial cells when grown in vitro conditions have a potential doubling time ranging between 2.4 and 13 days, whereas the turnover time in normal tissue is between 47 and 23,000 days (57). Hence, the tumor endothelial cells are more likely to be affected by treatment than the endothelial cells in normal tissues.

LLC exhibits a stabilizing profile at lower concentrations (5-100 nmole/ml), the cell line shows a cytotoxic effect with higher concentrations (≥ 100 nmole/ml). The effect of colchicine is cell line dependent, and interestingly we were not able to determine the IC$_{50}$ of LLC or MS1 VEGF with colchicine at the concentration used as we observed percent cell viability no less than 60%.

Colchicine can be incorporated into the PCLs with high efficiency owing to its lipophilic character (fig-1). The decrease in percent cell viability of LLC and MS1-VEGF was significant following treatment with liposomal colchicine at most of the concentrations evaluated when compared to the free drug. The liposomal formulation of colchicine (50 nmole/ml) was used for the qualitative studies as it is already reported that 50 nmole/ml colchicine brings about mitotic arrest in almost all the cells treated (18). It is obvious from the images acquired by fluorescence microscopy that the disruption of microtubules is significantly higher when the cell lines are treated with liposomal colchicine when compared to the free drug treatment. This indicates that the PCLs are undoubtedly increasing the concentration of colchicine taken up by the cell as the maximum cytoskeletal damage is observed with liposomal drug. A plausible explanation for this behavior might be that the concentration of colchicine taken up by cells during the free drug treatment was just enough to arrest the mitosis of some cells, whereas with liposomal drug treatment the concentration was sufficient to arrest all the cells resulting
in a significant decrease in the percent of cell viability. The significant decrease in the nuclear area in both the cell lines also suggests that liposomal colchicine is carried to the nuclei of the cells in higher concentrations compared to free colchicine. This resulted in a decrease in the total nuclear area, further supporting the notion that higher concentration of colchicine (50 nM) induces c-mitosis in the cells. C-mitosis is characterized by the cell division taking place in the absence of the spindle apparatus. This is followed by a breakdown of the nuclear envelope, condensed chromosomes, and undivided centromeres (18).

6.1 Influence of Cellular properties on the effect of PCLs

From the fluorescence microscopy studies it is evident that the PCLs are more effective against MS1-VEGF when compared to LLC. We have already observed that the cytoskeleton area for the MS1-VEGF cells (10645 ± 1199) is almost twice that of the cytoskeleton area of LLC (4554 ± 748.5). This observation supports the notion that the surface area of the endothelial cell is larger than the cancer cell, and hence, liposomes accumulate to a more significant extent in the endothelial cells. From the cell association studies we conclude that there is significantly higher uptake of PCLs by MS1-VEGF compared to the LLC cells, and that the cell size of MS1-VEGF as reported earlier is larger than LLC cells. Also, as stated earlier the expression of negatively charged moieties on tumor endothelial cells is 15-33 times higher when compared to the normal endothelial cells (56). All the above properties of the endothelial cells make them more susceptible to the effects of PCL-colchicine. The above mentioned properties of endothelial cells rationalize our choice of targeting the tumor vasculature as a drug.
delivery approach against Lewis lung carcinoma. Several lines of evidence suggests that colchicine-loaded PCLs can be used as a chemotherapeutic formulation, which potentially could be injected IV to reduce the toxic effects associated with the treatment.

7. Concluding remarks to date:

As discussed in previous sections, colchicine has demonstrated significant antivascular effects on human tumors, but associated toxic effects have prevented its use for anticancer (systemic) treatment. We thus formulated colchicine-loaded PCLs with high incorporation efficiency and determined the percent of cell viability using in vitro models. We conclude that the effect of free drug is cell line-dependent. We show that colchicine has a stabilizing effect on cells and that the liposomal drug activity was significantly higher when compared to free drug. We also conclude that the disruption of microtubules as well as the reduction in nuclear area was more significant with the use of liposomal colchicine. We also assume that the endothelials cells in vivo will benefit the most with liposomal drug treatment, due to the significantly greater cytoskeletal area when compared to cancer cells.

Though in vivo use of colchicine is not the goal of this study, we believe that these studies support our hypothesis that there is an advantage of using liposomal colchicine over the free drug for targeting the tumor vasculature. The exact mechanistic pathway of how colchicine can act as a vascular disrupting agent is yet to be determined.
8. Future Implications

We further aim to investigate the potential mechanisms involved in the complete vascular shut down \textit{in vivo} by using the \textit{in vitro} models. Herein we aim to address the mechanism(s) relating tumor vascular shut down with treatment. As discussed in the above sections, e-selectin (located on the surface of endothelial cells lining the tumor vasculature) is upregulated by cytokines. We are interested in determining if colchicine shows similar effects on activated endothelial cells as observed with normal endothelial cells in a resting state.
9. References:

1. The American Heritage® Dictionary of the English Language, fourth edition


