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TITLE: Procoagulant effects of lung cancer chemotherapy on HUVEC, A549 cells, and monocytes.

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## ABSTRACT

Cancer patients undergoing chemotherapy have an elevated risk for thrombosis. Although thrombosis is a common complication in cancer patients, the mechanisms of chemotherapy-induced thrombosis remain unclear. We investigated the procoagulant effects of lung cancer chemotherapy agents (carboplatin, paclitaxel, cisplatin, and gemcitabine) on endothelial cells, A549 cells, and monocytes. We also investigated the *in* vivo procoagulant effects of the aforementioned chemotherapeutic agents as well as the anti-angiogenic agent bevacizumab. Tissue factor (TF) activity, TF antigen and phosphatidylserine (PS) levels were measured on chemotherapy-treated human umbilical vein endothelial cells (HUVEC), A549 cells, and monocytes. Treatment of HUVECs, A549 cells, and monocytes with lung cancer single agent and combination chemotherapy resulted in significant increases in TF activity. However, only cisplatin- and gemcitabine-treated monocytes were found to have increased TF antigen levels. PS exposure was increased only on HUVEC and monocytes treated with cisplatin/gemcitabine combination therapy. Interestingly, addition of paclitaxel to carboplatin resulted in reduced levels of PS exposure on monocytes. This study is the first to explore the procoagulant effects of lung cancer chemotherapy agents on monocyte and A549 cell TF activity levels, as well as to investigate the mechanisms by which lung cancer agents may promote TF decryption on these cell lines**.** Our *in vivo* results demonstrated that treatment of healthy mice with bevacizumab, paclitaxel and carboplatin moderately increased plasma TAT levels in healthy mice. These studies reveal potential mechanisms by which lung cancer

chemotherapy may increase the risk of thrombosis. These studies reveal potential mechanisms by which lung cancer chemotherapy agents induce a hypercoagulable state.

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## LIST OF ABBREVIATIONS

II – prothrombin

IIa – thrombin

ADP – adenosine diphosphate

APC – activated protein C

ASCO – American society of clinical oncology

AT – antithrombin III

BSA – bovine serum albumin

$CaCl_2$ – calcium chloride

Cmax – maximum concentration

CP – cancer procoagulant

DNA – deoxyribonucleic acid

DVT – deep vein thrombosis

ELISA – enzyme-linked immunosorbent assay

EPCR – endothelial protein C receptor

F – factor

FBS – fetal bovine serum

FITC – Fluorescein isothiocyanate

Gla – γ carboxyglutamic acid

HUVEC – human umbilical venous endothelial cell

LPS – lipopolysaccharide

MACS – magnetic activated cell sorting

MP – microparticle

NSCLC – non-small cell lung cancer

PAR – protease-activated receptor

PBS – phosphate buffered saline

PC – protein C

PDI – protein disulfide isomerase

PS – protein S

RBC – red blood cell

RPMI – Roswell Park Memorial Institute growth medium

SE – standard error

sEPCR – soluble endothelial protein C receptor

SERPIN – serine protease inhibitor

sTM – soluble thrombomodulin

TAT – thrombin-antithrombin

TF – tissue factor

TFPI – tissue factor pathway inhibitor

TM - thrombomodulin

TNF – tumor necrosis factor

VEGF – vascular endothelial growth factor

VEGFR – vascular endothelial growth factor receptor

VTE – venous thromboembolism

vWF – von Willebrand Factor

## DECLARATION OF ACADEMIC ACHIEVEMENT

Zakhar Lysov contributed to conception and design of studies, performed all experiments, analyzed and interpreted the data, and performed statistical analyses.

Dr. Patricia C. Liaw contributed to conception and design of studies, obtained funding to support the studies, and critically reviewed the obtained results.

# 1.0 Introduction

## 1.1 Hemostasis

Hemostasis, the arrest of blood flow, is achieved through activation of the coagulation pathway. The coagulation pathway is a delicately balanced system that under normal function maintains blood in a fluid state within the vasculature. Blood fluidity is maintained through a dynamic equilibrium between clot formation and clot lysis. Upon vascular damage this system is able to rapidly form a clot and seal the injury to prevent excessive blood loss. In order to achieve an adequate blood clot in a timely manner, the coagulation system relies on numerous enzymatic reactions between clotting factors, cell associated cofactors and receptors, and plasma coagulation protease inhibitors. Furthermore, the coagulation pathway factors act in concert with the endothelium and platelets with the end result leading to fibrinogen cleavage by thrombin in order to form a fibrin clot (Mann et al. 2003; Mackman et al. 2007). When the hemostatic balance is lost, inadequate or excess clotting can lead to hemorrhage or thrombosis respectively.

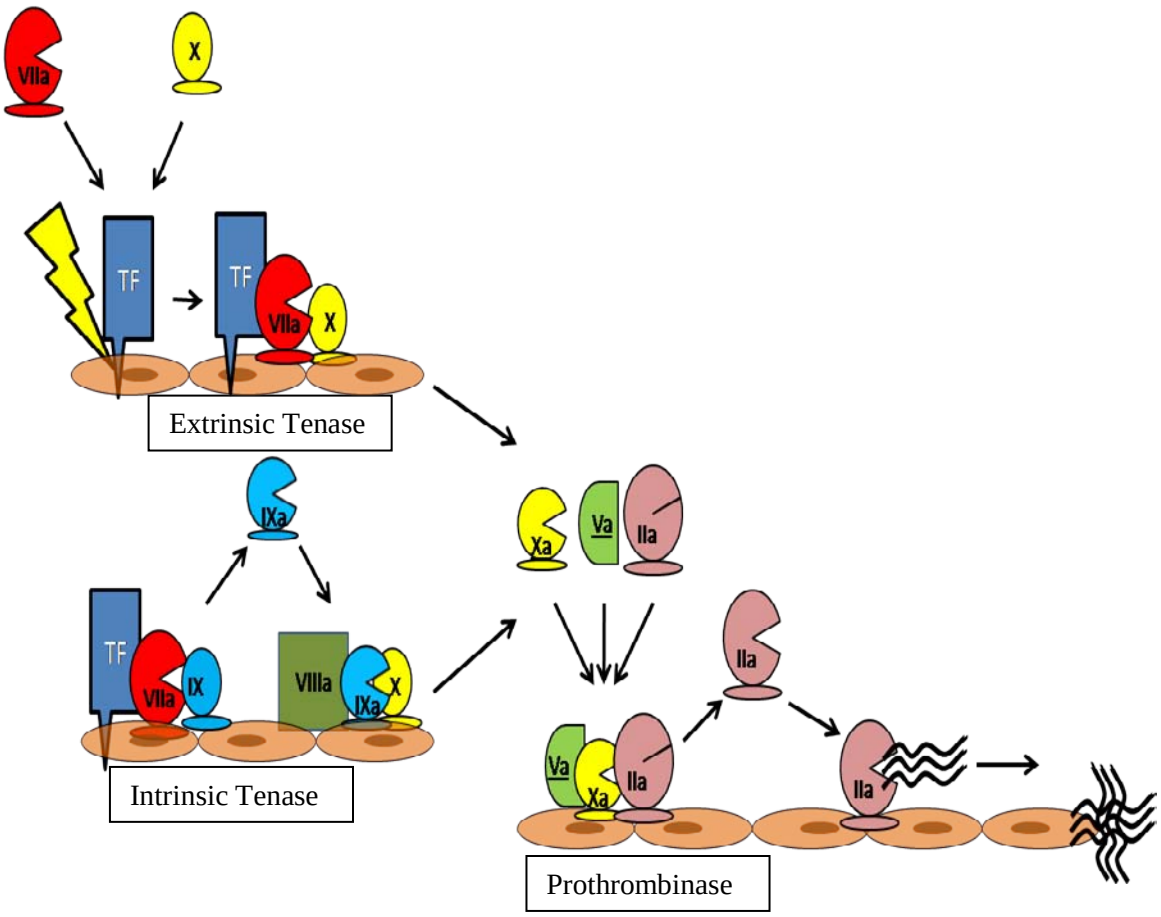
Vessel wall integrity is essential for prevention of thrombosis. Upon vascular wall damage, subendothelial von Willebrand Factor (VWF) and collagen exposure promotes platelet adhesion and activation at the site of injury (Wilner et al. 1968; Ruggeri 2003). Activated platelets release adenosine diphosphate (ADP) promoting further platelet activation and aggregation effectively forming an initial seal and preventing excess blood loss (Hovig 1963; Wagner and Burger 2003; Li et al. 2010).

Activation of the intrinsic or extrinsic pathway of the coagulation cascade is imperative to attain adequate clot formation. Initiation of the extrinsic pathway of

coagulation is triggered by exposure of tissue factor (TF) to blood (Dahlback 2005). Under physiologic conditions, TF is absent on endothelial cells but is constitutively expressed on extravascular cells found in the sub-endothelial layer (Monroe and Key 2007; Bluff et al. 2008). Thus, TF exposure to blood is usually a result of endothelial damage (Milsom and Rak 2008). Upon exposure to blood, TF forms a complex with circulating activated factor VII (FVIIa) forming the TF-FVIIa complex (Milsom and Rak 2008). The TF-VIIa complex in the presence of a phospholipid surface and $Ca^{2+}$ is then able to convert factors IX (FIX) and X (FX) into their active forms FIXa and FXa, respectively (Dahlback 2008).

**Figure 1. The coagulation pathway.**

Blood coagulation is initiated upon exposure of tissue (TF) to blood usually as a result of vascular injury. Exposed TF binds circulating factor VIIa (FVIIa) forming the extrinsic tenase complex. The newly formed TF-FVIIa complex activates FIX and FX into FIXa and FXa, respectively. FXa and its cofactor FVa convert prothrombin into thrombin. The resulting burst of thrombin acts in a positive feedback mechanism and further amplifies its own generation through activation of FV and FVIII. FXa generation can also occur via FIXa and its cofactor FVIIIa, generating additional thrombin, which in turn is able to convert fibrinogen into fibrin.


VIIa
X
TF
TF
VIIa
X
Extrinsic Tenase
IXa
TF
VIIa
IX
VIIIa
IXa
X
Intrinsic Tenase
Xa
Va
IIa
IIa
Va
Xa
IIa
IIa
Prothrombinase

Alternatively, coagulation can be initiated in the absence of the TF-FVIIa complex through the intrinsic or "contact" pathway. In the intrinsic pathway, circulating FXII is activated through contact with activated endothelium or the platelet surface, high molecular weight kininogen, kallikrein, polyphosphates, and collagen (Renne et al. 2005; Smith et al. 2006; Gailani and Renne 2007). Through positive feedback, activated FXII (FXIIa) cleaves prekallikrein to kallikrein, resulting in amplification of FXIIa generation (Muller et al.). FXIIa activates FXI (FXIa) which in turn converts FIX into its active form, FIXa (Gailani and Renne 2007). The resulting FIXa, in the presence of its activated cofactor VIII (FVIIIa) is also able to activate FX (Venkateswarlu et al. 2002; Gailani and Renne 2007).

FXa generated by the TF-FVIIa complex binds to FVa. The complex consisting of FXa, FVa, $Ca^{2+}$ and a negatively charged phospholipid surface is known as the prothrombinase complex. The prothrombinase complex is essential for cleaving the zymogen prothrombin (II) into thrombin. This initial burst of thrombin generated by the prothrombinase complex participates in a positive feedback mechanism generating increased amounts of FVa and activated FVIII (FVIIIa) (Fay 1988; Mann and Kalafatis 2003), leading to additional FXa and thrombin generation produced (Mann et al. 2003; Dahlback 2008; Milsom and Rak 2008) (Figure 1).

The formation of thrombin has numerous pro- and anticoagulant functions. Thrombin exerts procoagulant effects through initiation of platelet activation (Covic et al. 2000), FV, FVIII, and FXI activation as well as through cleavage of soluble fibrinogen into insoluble fibrin monomers necessary to form a fibrin clot (Milsom and Rak 2008).

Thrombin exerts anti-coagulant effects through binding to thrombomodulin (TM), a thrombin receptor found on the surface of endothelial cells (Esmon 2006). Upon binding to TM, thrombin indirectly exerts anticoagulant effects on the intrinsic and common pathways through activation of protein C (PC).

## 1.2 Tissue Factor

### 1.2.1 The tissue factor structure and pathway

Tissue factor, which is a 47 kDa transmembrane glycoprotein that consists of extracellular (residues 1-219), transmembrane (residues 220-242), and cytoplasmic (residues 243-263) domains (Chen et al. 2006), is the main initiator of coagulation (Broze 1982; Bach 1988; Wolberg et al. 1999). However, an alternatively spliced form of TF that lacks the transmembrane domain but retains cofactor activity has been described in humans and mice (Bogdanov et al. 2003; Bogdanov et al. 2006).

TF is constitutively expressed on fibroblasts and smooth muscle cells in the adventitia and medial blood vessel layers respectively (Mandal et al. 2006). Upon exposure of TF to blood, TF acts as a high affinity receptor for FVII and FVIIa (Broze 1982; Bach 1988; Wolberg et al. 1999), resulting in the formation of the extrinsic tenase complex (consisting of TF, FVIIa, $Ca^{2+}$ and a phospholipid surface), and initiation of the coagulation system. The formation of the extrinsic tenase complex enhances FVIIa catalytic activity towards FX by approximately 10,000 – fold (Bom and Bertina 1990).

Under physiologic conditions, vascular and intravascular cells such as endothelial cells and monocytes do not express cell surface TF (Drake et al. 1989). Endotoxins and

cytokines such as lipopolysaccharide, monocyte chemoattractant protein-1, and tissue necrosis factor can induce TF expression on endothelial cells and monocytes (Abe et al. 1999).

### 1.2.2 Tissue factor procoagulant activity

Although TF expression can be induced on the surface of monocytes and endothelial cells, changes in cell surface TF antigen levels do not always correlate with the changes in TF procoagulant activity levels. As a result of this dissociation between antigen and activity levels, two distinct populations of TF have been identified. Cell surface TF that possesses procoagulant activity is termed active/decrypted TF, whereas TF that does not possess procoagulant activity is termed inactive/cryptic TF (Bach 2006). Decryption of inactive TF has been attributed to mechanisms modulated by phosphatidylserine exposure (Bach 2006), protein disulphide isomerase (PDI) activity (Chen et al. 2006) or TF self association (Monroe and Key 2007).

### 1.2.3 Protein disulphide isomerase mediated tissue factor decryption

PDI, which is a chaperone protein found primarily within the endoplasmic reticulum, is essential for disulphide bond formation and isomerization (Freedman et al. 1994). However, PDI has also been shown to be expressed on the surface of endothelial cells where it associates with cell surface TF (Ahamed et al. 2006). One postulated PDI dependent mechanisms for TF decryption involve the formation of a disulphide bond between Cys186 and Cys209 (Pendurthi et al. 2007).

Recent studies have demonstrated that cryptic TF contains unpaired Cys186 and Cys209 residues located in the extracellular domain (Chen et al. 2006). Furthermore, mutation of these cysteines residues to serine has been shown to impair TF procoagulant activity (Rehemtulla et al. 1991). Moreover, thiol oxidizing agents increase TF activity in a time- and dose-dependent manner (Chen et al. 2006; Pendurthi et al. 2007). Because the formation of the disulphide bond is thought to induce a conformational change in TF, a monoclonal antibody specific to residues 181-214 of the human TF protein (V1C7) (Magdolen et al. 1998) was used to probe for this conformational change as a result of the Cys186-Cys209 disulphide bond formation. These studies revealed increased binding of V1C7 antibody to cells stimulated with thiol oxidizing agents compared with unstimulated cells (Chen et al. 2006).

It is important to note that conflicting results have been published on PDI mediated TF decryption mechanisms. One group hypothesized that increased TF activity on cells stimulated with $HgCl_2$ was due to increased PS exposure and not disulphide bond formation. Supporting experiments demonstrated reduced TF activity after annexin V addition and lack of PDI localization to the cell surface of $HgCl_2$ treated MDA-MD-231 cells (Pendurthi et al. 2007). More importantly, a recent study demonstrated that treatment of HUVEC expressing mutant TF lacking either the Cys186, Cys209 or both with thiol oxidizing agents, produced TF activity similar to that of wild type TF (Pendurthi et al. 2007). This study however did not address the effect of these thiol oxidizing agents on the HUVEC phospholipid environment. Furthermore, modulation of extracellular PDI was reported to affect PS asymmetry (Popescu et al. 2010). Due to

extensive roles of PDI in the cellular environment, the exact contribution of PDI to TF decryption remains unknown. Therefore, further investigation and clarification are necessary in order to address these inconsistencies.

### 1.2.4 Phosphatidylserine mediated tissue factor decryption

Phosphatidylserine is a negatively charged phospholipid is sequestered on the inner leaflet of the cell membrane of quiescent cells (Zwaal and Schroit 1997) by the action of two lipid translocases; flippase and floppase. Flippase is responsible for the inward translocation of phosphatidylserine, whereas floppase is responsible for outward translocation (Zwaal et al. 2005). The association between phosphatidylserine and TF procoagulant activity has been shown in a number of studies which demonstrate parallel increases in phosphatidylserine exposure occurring and TF activity (Zwaal and Schroit 1997; Lentz 2003).

Phosphatidylserine exposure and TF activity are both $Ca^{2+}$ dependent processes. Calcium ionophore treatment of cells causes an influx of extracellular $Ca^{2+}$ across the cell membrane triggering a disruption of phosphatidylserine asymmetry at the same magnitude of $Ca^{2+}$ influx that causes TF activity to be expressed (Bach 2006). This effect is attributed to the inhibition of floppase activity by large increases in cytosolic $Ca^{2+}$ levels causing the outward translocation of phosphatidylserine to the outer leaflet of the cell membrane (Zwaal et al. 2005). Furthermore, the inhibition of phosphatidylserine by Annexin V binding on calcium ionophore-stimulated cells also inhibits TF activity (Wolberg et al. 1999).

### 1.2.5 Evidence for TF self association model

The self association model of TF encryption/decryption postulates that cryptic pools of extracellular TF are present as homodimers, whereas decrypted TF pools exist as monomers (Roy et al. 1991). Evidence supporting this model demonstrates chemical cross linking between TF monomers (Roy et al. 1991). It is also important to note that cross linking of TF on cell surface can be prevented with calcium ionophore stimulation (Bach and Moldow 1997). The ability of intracellular changes in $Ca^{2+}$ levels to prevent TF dimerization and induce changes is phosphatidylserine exposure asymmetry suggests that this model of TF decryption is correlated with the phosphatidylserine induced TF decryption model. Therefore, calcium ionophore-induced disruption in phosphatidylserine asymmetry and reduction of TF dimerization may not be mutually exclusive.

### 1.2.6 Tissue factor in cell signaling

Although TF is the main initiator of the extrinsic coagulation pathway, it has also been shown to play important roles in cell signaling pathways via activation of G-protein coupled protease activated receptors (PARs) (Morrissey 2001). Moreover, TF is essential for embryonic survival and development. Thus TF null mouse embryos die in utero due to vascular abnormalities (Toomey et al. 1996). Studies also demonstrate increased cytosolic levels of $Ca^{2+}$ upon FVIIa binding to TF expressing cells (Rottingen et al. 1995), suggesting that TF participates in numerous signaling roles, including angiogenesis (Carmeliet et al. 1997), and growth and metastasis (Morrissey 2001). In fact, when tumor cells that overexpress TF are implanted into mice, larger and more vascularized tumors develop compared with control tumor cells (Zhang et al. 1994).

### 1.3 Regulation of Coagulation

In order to prevent adverse clotting, a number of endogenous anticoagulant mechanisms exist to attenuate the coagulation pathway. Antithrombin (AT) (Rau et al. 2007), tissue factor pathway inhibitor (TFPI), (Baugh et al. 1998) and protein C (PC) (Regan et al. 1994; Egan et al. 1997; Esmon 2003) all have distinct roles in attenuating the coagulation pathway to ensure proper hemostasis.

#### 1.3.1 Antithrombin

AT is a serine protease inhibitor (serpin) that inhibits the coagulation pathway through formation of a 1:1 complex with the majority of coagulation enzymes (Rau et al. 2007). It primarily inhibits thrombin and FXa and to a lesser extent FIXa, FXIa, and FXIIa (Hirsh et al. 2001). Inhibition of thrombin by AT alone is slow and inefficient. However, in the presence of its cofactor heparin (Hirsh et al. 2001) or heparan sulfate (Lindahl 2007), the rate of thrombin inhibition by AT is increased by ~4000 fold (Olson et al. 1992).

The observed effect of heparin as a cofactor for AT depends on a unique pentasacharide sequence (Hirsh et al. 2001; Gomez et al. 2005). The interaction between AT and the unique heparin pentasaccharide sequence induces a conformational change in AT that increases the ability of AT to bind and inhibit FXa (Gomez et al. 2005). Thrombin inhibition is increased by AT in the presence of heparin containing a minimum of 13 saccharide units in addition to the unique pentasaccharide sequence. This facilitates the alignment and interaction of the two molecules and increases protease inhibition by AT (Hirsh et al. 2001).

### 1.3.2 Tissue factor pathway inhibitor

TFPI is a proteinase inhibitor located primarily on the endothelium and to a lesser extent on vascular smooth muscle cells (Crawley et al. 2000). TFPI consists of three tandem Kunitz type domains and forms a quaternary complex that effectively inhibits the TF-VIIa-Xa complex (Hamamoto et al. 1993). The formation of the quaternary structure occurs with the binding of FVIIa and FXa to TFPI Kunits type domains 1 and 2 respectively (Baugh et al. 1998). Although the function of TFPI Kunitz type domain 3 remains unclear, it is believed to play a role in binding to glycosaminoglycans (Bajaj et al. 2001).

While the majority of TFPI is localized on the surface of endothelial cells, concentrations of TFPI can be increased by administration of heparin. This causes TFPI displacement from the surface of endothelial cells and results in up to a 3-fold increase in plasma TFPI concentration (Brodin et al. 2004; Mann et al. 2006).

### 1.3.3 Protein C pathway

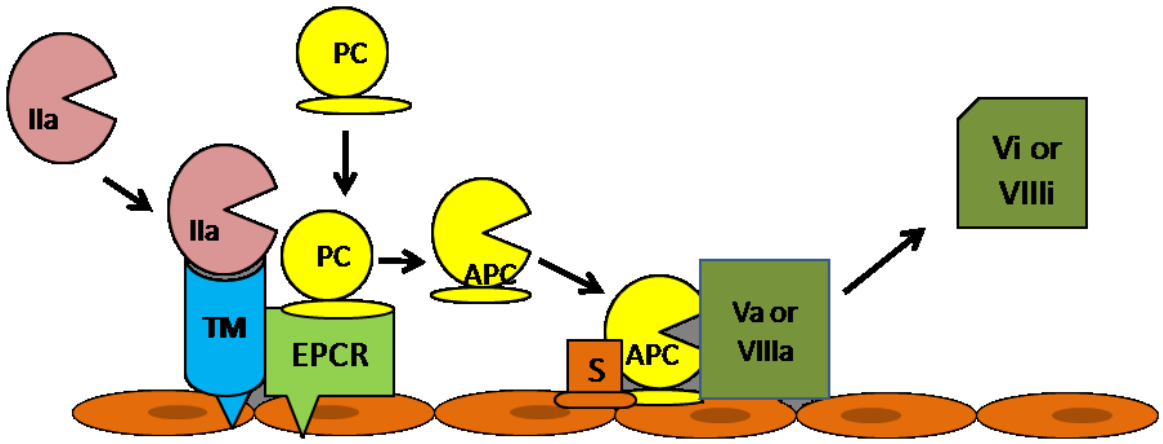
Protein C is synthesized in the liver as a zymogen precursor to activated protein C (APC), a key molecule that has multifunctional hemostatic and anti-inflammatory roles (Egan et al. 1997; Levi et al. 2003). Although thrombin is capable of converting PC to APC, this reaction does not occur at an adequate rate to be physiologically relevant (Esmon et al. 1982). PC and thrombin bind to endothelial protein C receptor (EPCR) and TM respectively, both of which are located on the endothelial cell surface. Thrombin bound to TM augments PC activation by 1000-fold (Esmon et al. 1982; Esmon 2006).

The binding of PC to EPCR further amplifies PC activation by approximately 20-fold (Taylor et al. 2001).

Upon activation, APC dissociates from EPCR and in the presence of the cofactor protein S, is able to attenuate thrombin generation through proteolytic cleavage of FVa (Egan et al. 1997) and FVIIIa (Regan et al. 1994) (Figure 2). However, PC, TM, and EPCR also play important hemostatic roles in embryonic development and survival. Ablation of TM or EPCR causes in utero death (Healy et al. 1995; Gu et al. 2002). While mice that lack PC survive to birth, death occurs within 24 hours of delivery due to coagulopathy (Jalbert et al. 1998).

**Figure 2. Protein C Pathway**

Once thrombin (IIa) is generated, it is able to bind to thrombomodulin (TM) on the surface of endothelial cells. The IIa-TM complex converts protein C (PC) into activated protein C (APC) through a single proteolytic cleavage and release of a 12-amino acid activation peptide. APC generation is enhanced by PC binding to endothelial protein C receptor (EPCR). APC in complex with the cofactor protein S (S) inactivates factors Va and VIIIa.


IIa
PC
IIa
PC
APC
TM
EPCR
S
APC
Va or
VIIIa
Vi or
VIIIi

### 1.4 Vascular Endothelium

The blood vessel lumen is surrounded by vascular endothelium, a single cell layer that is crucial for maintaining blood fluidity. The endothelial cell layer acts as a physical barrier between flowing blood and the subendothelial layers that constitutively express TF (Mandal et al. 2006). Because TF is absent on the endothelium under physiologic conditions, exposure of TF to blood is usually a direct result of vascular damage (Drake et al. 1989).

Upon adequate formation of a fibrin clot due to injury, the coagulation process must be terminated in order to prevent thrombus formation on adjacent, uninjured endothelium. In order to limit coagulation, the protein C pathway is activated in response to initial thrombin generation on intact endothelial cells expressing EPCR and TM. Inactivation of FVa and FVIIIa by APC is amplified approximately 10-fold in the presence of protein S, a soluble cofactor for APC (Walker 1980). Once thrombin is generated at the site of injury reaches an intact endothelial cell, it binds to TM to facilitate the activation of PC bound to EPCR. The newly activated PC along with its cofactor protein S is then able to proteolytically inactivate factors Va and VIIIa effectively terminating generation of any additional thrombin. In addition to providing a surface for APC generation, displacement of endothelial cell-bound TFPI by heparin results in decreased thrombin generation by the formation of TF-FVIIa-TFPI-FXa complex.

Furthermore, endothelial cells are a source of prostacyclin and nitric oxide, release of which promotes vasodilation and inhibition of platelet activation (Wang et al. 1998; Becker et al. 2000). Platelet aggregation is further inhibited through the production of

endothelial ecto-ADPase, which metabolizes platelet derived ADP (Marcus et al. 1997). The endothelium also plays a role in fibrinolysis through the release of tPA and uPA (Levin and del Zoppo 1994; Levin et al. 1997; Takahashi et al. 1998).

### 1.5 Cancer and thrombosis

As a result of anti-cancer therapies, indwelling catheters, type of cancer, and disease progression, cancer adversely impacts coagulation and has been associated with an increased incidence of thrombotic events. In fact, cancer patients have been reported to have an overall 5-fold higher incidence rate of developing a venous thromboembolism (VTE) compared with the healthy population (Sud and Khorana 2009). In fact, 1 in 7 cancer patient die from pulmanory embolism, making thrombosis the second leading cause of death amongst cancer patients (Ambrus et al. 1975).

While numerous individual factors associated with cancer-induced thrombosis have been outlined, the mechanism(s) by which cancer increases the thrombotic risk are not fully understood. Cancer cells have been shown to express TF (Koomagi and Volm 1998; Ueno et al. 2000) and cancer procoagulant (CP) (Falanga and Gordon 1985) on the cell membrane. Expression of TF has been reported on numerous types of cancers including pancreatic, lung, and colorectal cancer cells and TF expression is associated with higher risk of metastasis and thrombosis (Rickles et al. 1995; Sawada et al. 1999; White et al. 2007). CP is a cysteine protease that has been shown to accelerate clotting times in FVII deficient plasma by directly activating FX (Gordon et al. 1975).

**Table 1. Properties of chemotherapy and anti-angiogenesis therapy agents**

| | Cisplatin | Gemcitabine | Carboplatin | Paclitaxel | Bevacizumab |
|---|---|---|---|---|---|
| **Target** | DNA | DNA | DNA | β-tubulin | VEGF |
| **Method of entry** | Copper Transporter 1 (CTR1) | Nucleoside Transporters | Copper Transporter 1 (CTR1) | Passive Diffusion | N/A |
| **Plasma Cmax** | 20 µM | 60 µM | 40 µM | 4 µM | 4 µM |
| **Mechanism of Action** | DNA Adduct | DNA Adduct | DNA Adduct | Microtubule Stabilization | VEGF Inhibition |

### 1.6 Chemotherapy and thrombosis

Lung cancer is the second most common type of cancer with non-small cell lung cancer (NCLC) accounting for 80% of all lung cancers (Onn et al. 2004; Jemal et al. 2010). The American Society of Clinical Oncology (ASCO) recommends platinum-based (cisplatin or carboplatin) combination chemotherapy over non-platinum based therapies for first line treatment of NSCLC (Azzoli et al. 2010). Cisplatin and carboplatin are commonly administered in combination with gemcitabine and paclitaxel, respectively, and have been studied extensively for their anti-tumor efficacy (Sandler and Ettinger 1999; Scagliotti et al. 2002; Sandler et al. 2006). Although it has been clinically recognized that treatment of cancer patients with chemotherapeutic agents results in increased incidence of (VTE), the molecular mechanisms by which these chemotherapeutic agents contribute to thrombosis are not fully understood.

Varying rates of thrombosis have been reported in the literature depending on the type of cancer present and the chemotherapy treatment utilized. For example, incidence rates of thrombosis in breast cancer patients undergoing chemotherapy vary between 5% and 17.6% (Weiss et al. 1981; Goodnough et al. 1984). In lung cancer patients undergoing chemotherapy, the incidence rate of thrombosis was reported to be as high as 27.9%, with thrombotic events occurring within 1 month of therapy initiation (Blom et al. 2004). For treatment of NSCLC, platinum-based combination therapies are often utilized to maximize the anti-tumor efficacy of the agents.

### 1.6.1 Mechanism of action of anti-cancer agents

The majority of chemotherapeutic agents elicit their anti-tumor effects by induction of apoptosis through irreversible DNA damage. Cisplatin and carboplatin are two platinum based agents that form platinum adducts with DNA strands after passive diffusion into the cells, thereby preventing DNA replication and cell division (Calvert et al. 1982; Jamieson and Lippard 1999; Numico et al. 2005). Other non-platinum based agents used for treatment of lung cancer, such as gemcitabine, employ a similar mechanism of action. Gemcitabine is a prodrug that must be phosphorylated into the active form upon entry into the cell (Plunkett et al. 1995). Paclitaxel, originally isolated from yew trees, is a compound that acts on the microtubule environment of the cell (Horwitz 1992). Paclitaxel is capable of stabilizing established microtubule environments as well as shifting soluble tubulin proteins into a stable microtubule polymer. Microtubules are in a dynamic state where tubulin is responsible for mitotic spindle formations, cell shape maintenance, and cell motility (Horwitz 1992). Stabilization of the microtubule environment would effectively disrupt normal cell activities as well as cell division.

Our greatest understanding of chemotherapy associated thrombosis comes from studies investigating the procoagulant effects of chemotherapeutic agents in used in the treatment of breast cancer. Previous studies in our lab demonstrated that treatment of endothelial cells with chemotherapy agents reduced the levels of EPCR (Woodley-Cook et al. 2006), which reduces the capacity for PC activation (Woodley-Cook et al. 2006; Swystun 2011). We and others have shown increased TF activity and enhanced thrombin

generation on chemotherapy treated endothelial cells (Ma et al. 2005; Swystun et al. 2009; Swystun 2011). Breast cancer patients undergoing chemotherapy were found to have decreased PC and protein S levels (Rella et al. 1996). In addition, increased incidence of thrombosis seen in cancer patients has been attributed to chemotherapy-induced microparticle shedding (Fu et al. ; Lechner et al. 2007).

Previous *in vitro* studies have shown that treatment of endothelial cells cisplatin and gemcitabine results in a dose-dependent increase of TF activity (Ma et al. 2005). However, studies investigating the effects of paclitaxel on endothelial cells have produced conflicting results. Wang et al. reported increased TF activity and antigen levels (Wang et al. 2009), whereas Napoleone et al. reported decreased TF activity (Napoleone et al. 2009).

### 1.6.2 Cisplatin

Cisplatin is a platinum based agent that upon entry into a cell, forms platinum adducts onto DNA strands. This in turn affects DNA replication leading to cell cycle arrest and apoptosis (Stordal and Davey 2007). Although increased rates of thrombotic events have been reported with cisplatin use, it is often used in combination with other chemotherapeutic agents making it difficult to identify the prothrombotic potential of cisplatin alone. When cisplatin is used as sole therapy, vascular events are seldom reported (Numico et al. 2005). *In vitro*, cisplatin upregulates TF activity and downregulates TFPI activity (Ma et al. 2005). Cisplatin has also been shown to enhance aggregation time of platelets in human platelet rich plasma (Togna et al. 2000).

### 1.6.3 Carboplatin

Carboplatin is a modified form of cisplatin with a similar mechanism of action but fewer toxic side effects (Calvert et al. 1982). Interestingly, increased rates of thrombocytopenia have been reported in patients receiving carboplatin. A number of individual clinical studies have compared cisplatin and carboplatin efficacy. A meta-analysis of nine studies examined cytotoxic profiles of cisplatin- or carboplatin-based combination therapy. Rates of thrombocytopenia in patients receiving carboplatin-based combination therapy were reported to be 2-fold higher in comparison to the cisplatin-based combination therapy (Ardizzoni et al. 2007).

### 1.6.4 Gemcitabine

Gemcitabine is a chemotherapeutic agent that is administered intravenously as either a single agent or as part of a platinum-based combination. It is a nucleoside analogue and therefore acts on newly dividing cells by being incorporated into the DNA, causing termination of DNA replication and disturbing the DNA integrity of the cell (Sandler and Ettinger 1999). One study investigating the efficacy of cisplatin/gemcitabine combination therapy reports vascular event (stroke, thrombosis, embolism etc.) occurrence in 22/108 patients treated, 12 of which were either a DVT or a pulmonary embolism (Numico et al. 2005). Only 1 DVT was reported in a phase III study that included 45 patients of patients receiving cisplatin/gemcitabine combination therapy. Furthermore, the report only took into consideration the first two cycles of treatment whereas each patient received an average of four cycles as part of their therapy (Scagliotti et al. 2002). In vitro studies investigating the procoagulant effect of gemcitabine have

found that treatment of endothelial cells with gemcitabine results in increased TF activity and decreased TFPI activity (Ma et al. 2005).

### 1.6.5 Paclitaxel

Paclitaxel (trade name Taxol) was originally derived from the bark of yew trees and exerts its anti-tumor effects via stabilization of the cellular microtubule environment (Horwitz 1992). Paclitaxel binds to β-tubulin, allowing for the formation but not degradation of microtubules. Due to cellular dependence on a dynamic microtubule environment for cell transport, motility, and cell division, stabilization of this dynamic environment is lethal to the cell (Nogales 2000). Divergent findings on the procoagulant effects of paclitaxel therapy have been reported. While one study demonstrated increased TF expression and activity on paclitaxel treated human aortic endothelial cells (Stahli et al. 2006; Wang et al. 2009), another study reported decreased TF levels on paclitaxel treated endothelial cells (Napoleone et al. 2009), suggesting that further studies are needed.

## 1.7 Anti-angiogenesis therapy

In 1989, a novel molecule was identified and labeled as vascular endothelial growth factor (VEGF) (Ferrara and Henzel 1989). Through retinal research, this molecule was recognized as the initiator of vascular remodeling and promoter of angiogenesis in areas of hypoxia (Stone et al. 1995). Tumor growth and viability relies on a well established vascular network to deliver essential nutrients to rapidly dividing cells (Hanahan and Folkman 1996). As a result of excessive proliferation, a growing tumor will outgrow the established vascular network and a hypoxic environment will be formed

when the metabolic needs of a malignant cell are not being met. The formation of novel vasculature from pre-existing blood vessels (angiogenesis) is required to re-establish an adequate nutrient supply. To achieve novel vasculature formation, malignant cells undergoing hypoxia will synthesize and secrete VEGF to induce endothelial cell proliferation and migration (Neufeld et al. 1999). The various isoforms of VEGF bind to VEGFR-1 (flt) and VEGFR-2 (KDR/flk-1) receptors, which are found on the surface of endothelial cells (Neufeld et al. 1999). The binding of VEGF to the extracellular region of VEGFR promotes receptor dimerization and initiates intracellular autophosphorylation which prompts angiogenesis (Keyt et al. 1996). This prompted scientists to target VEGF as well as the associated receptors in search of a novel anti-cancer therapy.

### 1.7.1Bevacizumab

Bevacizumab (trade name Avastin) is a monoclonal antibody that was approved for treatment of lung cancer in 2006 (Folkman 2007). The mechanism of action is through binding and neutralizing the VEGF-A isoform (Presta et al. 1997). Conflicting results have been published as to whether bevacizumab significantly increases thrombotic events. Although found to not be statistically significant, thrombotic events occurred in 19.4% of the patients receiving bevacizumab compared with 16.2% of patients who were not (Hurwitz et al. 2004) and 12.5% -17.6% of bevacizumab arm (depending on dose) compared to 9.4% that is seen in the control arm (Johnson et al. 2004).

### 1.8 Aims and Hypothesis

The primary evaluation and success of chemotherapeutic and anti-angiogenesis drugs is based on their efficacy on tumor regression and progression free survival. Combination lung cancer chemotherapy has proven to be more beneficial in median and 1-year survival times compared to single agent therapies (Lilenbaum et al. 2005). Moreover, further increases in median and progression free survival have been reported in lung cancer patients receiving anti-angiogenesis therapy adjuvant to chemotherapy (Sandler et al. 2006). As a result of ASCO recommendations, platinum-based combination therapy consisting of two or more chemotherapeutic agents has become common practice as first line chemotherapeutic defense in NSCLC lung cancer therapy (Azzoli et al. 2009).

While administration of combination chemotherapy has proven to be successful for anti-cancer treatments, the procoagulant side effects and increased thrombotic events associated with anti-cancer agent use have also been reported (Heit et al. 2000; Johnson et al. 2004; Numico et al. 2005). The overall objective of this project is to determine the mechanisms by which chemotherapeutic and antiangiogenic agents increase the risk of thrombosis in lung cancer patients.

**We hypothesize that the aforementioned chemotherapy and anti-angiogenesis therapy agents will affect the endothelial cell, A549 NSCLC and monocyte hemostasis by (A) up-regulating tissue factor activity and/or antigen levels and (B) increasing cell surface phosphatidylserine levels.**

We examined the effect of lung cancer chemotherapeutic agents on (a) cell surface tissue factor activity and antigen, (b) on mechanisms of tissue factor decryption, and (c) procoagulant activity *in vivo*.

Our specific aims to investigate our hypotheses were:

**Aim 1:** To examine the effects of lung cancer chemotherapy agents on cell surface TF activity of HUVEC, A549 cells, and monocytes.

**Aim 2:** To investigate the effects of lung cancer chemotherapy agents on cell surface TF antigen and phosphatidylserine exposure on HUVEC, A549 cells and monocytes.

**Aim 3:** To determine the effects of lung cancer chemotherapy agents on mechanisms of TF decryption.

**Aim 4:** To **s**tudy the procoagulant effects of lung cancer chemotherapy and anti-angiogenesis agents *in vivo.*

## 2.0 Experimental Methods

### 2.1 Materials

Human umbilical vein endothelial cells (HUVECs) and endothelial growth medium (EGM-2MV) bullet kits were purchased from Lonza (Basel, Switzerland). A549 non-small cell lung cancer cells were purchased from ATCC (Manassas, VA, USA). Heparin was purchased from Leo Pharma, Inc. (Thornhill, ON). RPMI 1640 growth medium, trypsin-EDTA and penicillin-streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS) and BSA were purchased from Sigma-Aldrich (St Louis, MO, USA). Anti-human TF monoclonal antibody (FITC) was from Affinity Biologicals (Ancaster, ON, Canada). Anti-PDI (RL90) was from AbCam, (San Diego, CA, USA). Annexin V, FITC- annexin V, Annexin V binding buffer, and anti-TF inhibitory antibody HTF1 were purchased from BD Biosciences (San Jose, CA, USA). Factors VIIa, FXa and FX were from Haematologic Technologies (Essex Junction, VT, USA). Chromogenic substrate S2765 was purchased from DiaPharma (West Chester, OH, USA). MACS columns and CD-14 microbeads were from Miltenyi Biotec (Auburn, CA, USA). Cisplatin was purchased from Mayne Pharma (Salisbury South, SA, USA). Carboplatin was purchased from Novopharm (Toronto ON). Paclitaxel was purchased from Bristol-Myers Squibb (New York City, NY. USA) and Gemcitabine was purchased from Ely Lilly and Company (Indianapolis, IN. USA). Bevacizumab (Avastin) was purchased from Genentech (San Francisco, CA. USA).

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**2.2 Cell Culture:**

HUVEC were cultured in flasks pre-coated with 2% gelatin between passages 1-4 in EGM-2MV medium. A549 cells were grown in flasks in RPMI 1640 supplemented with 10% FBS and 1% penicillin-streptomycin. Growth medium was changed every 2-3 days and new medium was used for treatment of cells with chemotherapeutic agents. Cells were harvested using 10X trypsin-EDTA and all experiments were conducted upon achieving 80-90% confluence.

**2.3 Monocyte Isolation:**

For monocyte collection, whole blood was collected into syringes prepared with 10 units of heparin per mL of blood. 25-30 ml of whole blood was layered on top of 15 ml of Histopaque 1077 and centrifuged at 1500 RPM for 30 minutes with the break off. Plasma was discarded and PBMC were transferred into a clean tube, washed in PBS and repelleted at 5000 RPM for 5 minutes. To eliminate red blood cells (RBC) contamination, the pellet was resuspended in RBC lysis buffer (155 mM $NH_4Cl$, 10 mM $KHCO_3$, and 0.1 mM EDTA) and incubated for 30 minutes at room temperature. The mixture was centrifuged again for 5 minutes at 1500 RPM and the pellet was visually inspected for RBC content, if RBC could be seen, the RBC lysis step was repeated at 10 minute incubation intervals. Upon achieving a RBC free pellet, the remaining cells were washed twice with PBS.

Isolation was done by resuspending the RBC free pellet in PBS (2.5ml PBS for every 50 ml of whole blood) and MACS anti CD-14 microbeads (2 µl/ml of whole blood) and allowed to incubate for 30 minutes at room temperature. During the incubation period, the LS column was washed with 10 ml of MACS buffer. The PBMC-CD14 microbead mixture was spun and the resulting pellet was washed twice with PBS, resuspended in 2-3 ml of MACS buffer and transferred to a LS column placed in a MACS multistand. Once the entire mixture ran through, the column was further washed with 15 ml MACS buffer. Lastly, 2-3 ml of MACS buffer was placed in the LS column and the cells were forced into a clean 15-ml falcon tube with the use of the plunger. The resulting monocyte population was washed with PBS and resuspended in complete RPMI at a density of $1x10^6$ cells. All cells were cultured at $37^oC$ in a 5% $CO_2$ environment.

**2.4 Concentrations of chemotherapeutic agents**

Cultured HUVEC, A549 cells, and isolated monocytes were treated with chemotherapy agents either as single agent treatment or in platinum-based combinations of cisplatin and gemcitabine (cisplatin/gemcitabine) or carboplatin and paclitaxel (carboplatin/paclitaxel). Concentrations of chemotherapeutic agents used were based on maximum plasma concentrations (Cmax) obtained from clinical studies of patients undergoing chemotherapy (Kern et al. 2001; Kroep et al. 2006).

Chemotherapy treatments were performed by addition of chemotherapeutic agents directly to the culture medium of the cells. Cells treated with combination therapy were exposed to both agents concurrently for 24 hours.

**2.5 Tissue factor activity Assay**

Cells were treated for 24 hours with single agent or combination chemotherapy and were washed twice with TBS. 300 uL of TBS containing 5nM of FVIIa and 150nM FX or 150 nM FX only, followed by 5mM $CaCl_2$ was added to each condition and allowed to incubate for 30 minutes at 37$^{o}$C. 250 uL of supernatant was transferred to a 96-well plate and FXa chromogenic substrate (S-2765) was added at a final concentration of 0.2 mM final. After 3 minute incubation at room temperature, the reaction was stopped with 20 uL of 50% acetic acid and the endpoint optical density value was read at 405 nm. Cells were removed from the wells using trypsin-EDTA and a cell count was performed on a hemocytometer. Doxorubicin treatment was used as a positive control. Concentration of FXa was determined from a previously established standard curve using known concentrations of FXa. TF and PDI inhibitory antibodies, HTF-1 and RL90 respectively were both used at a final concentration of 10 ug/mL. TF inhibition was achieved by incubating chemotherapy treated cells for 30 minutes with HTF-1 at room temperature prior to initiation of TF activity assay. PDI inhibition was achieved by incubating cells with RL90 for 30 minutes at room temperature prior to exposure to chemotherapy agents. PDI inhibitory antibody was dialyzed in PBS overnight using dialysis cassettes.

## 2.6 Flow Cytometry

### 2.6.1 Cell surface TF antigen Levels

HUVEC, monocytes, and A549 cells treated for 24 hours with combination therapy were harvested using trypsin-EDTA and transferred into 1.5 mL eppendorf tubes. Cells were pelleted by centrifugation at 4000 rpm for 5 minutes and the supernatant was discarded. The pellet was washed and resuspended in PBS. Fluorescent mouse anti-human TF monoclonal antibody was added using a final concentration of 2 ug/mL to label cell surface TF. The cells were allowed to incubate for 30 minutes with agitation at room temperature in the dark. The cells were then spun down and supernatant was discarded. The pellet was resuspended in 200 uL of PBS and the geometric mean of cell fluorescence was measured by flow cytometry. TNFa was used as a positive control for cell surface TF antigen level increase. Monocytes underwent an additional blocking step in order to prevent non-specific IgG binding to the Fc receptors. Monocyte Fc receptors were blocked using 200 ug/ml ChromPure Human IgG on ice for 1 hour prior to addition of fluorescent antibody

### 2.6.2 Cell surface phosphatidylserine exposure

Phosphatidylserine labeling was done according to the technical data sheet supplied with the FITC-Annexin V by BD Biosciences. Briefly, treated cells were harvested using trypsin-EDTA and transferred to eppendorf tubes. The cells were spun down at 4000 rpm for 5 minutes and washed twice with PBS. The pellet was resuspended in 100 uL of Annexin V binding buffer, labeled with 5 uL of FITC-Annexin V and

allowed to incubate at room temperature in the dark for 15 minutes. The fluorescence was measured by flow cytometry. Cells treated with 0.1% $H_2O_2$ for 4 hours were used as a positive control.

**2.7 TAT complex formation in murine plasma**

Mice were injected intraperitoneally with chemotherapy and anti-angiogenesis therapy agents on days 1, 2 and 3. Blood was collected Plasma concentrations of TAT complexes were measured using the Enzygnost TAT micro kit. Mice were injected once on three consecutive days and blood was collected into citrated syringes on day 4 (24 hours post last injection). Blood was collected using the carotid cannulation method into citrated syringes and double spun at 5000 RPM for 5 minutes as previously described (Gross et al. 2005). Briefly, mice are anesthetized using a Ketamine/Xylazine/Atropine cocktail injected intraperitoneally. The carotid artery is then isolated and a tourniquet is applied at the distal end of working region. To prevent bleeding, the proximal end of the carotid artery is temporarily clamped and a small incision is made between the clamp and the tourniquet. Cannulas is inserted proximally and the clamp is removed to allow blood flow. An aliquot of the plasma was transferred into a new eppendorf tube and stored at -80$^{o}$C for later analysis. These experiments have been approved by McMaster's Animal Research Ethics Board.

### 2.8 Statistical Analysis

Values are expressed as means ± standard error. Statistical analysis was performed using one way ANOVA, Tukey's pair wise comparisons or t-tests on SigmaPlot software version 11.2. All results are representative of at least 3 individual experiments. Figures denote $p < 0.05$ with *, $p < 0.01$ with **, and $p < 0.001$ with ***.

## 3.0 Results

### 3.1 Effects of platinum-based chemotherapy on TF activity

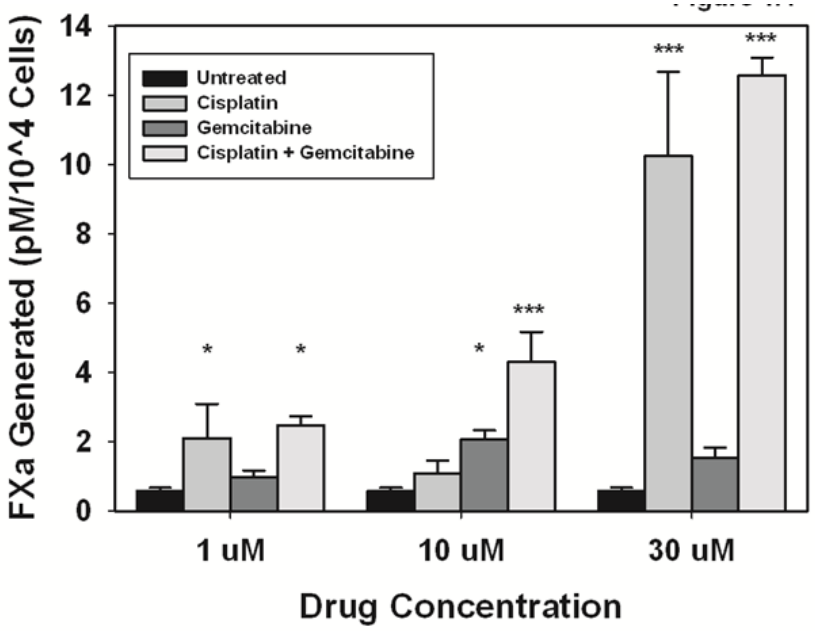
To examine the mechanisms by which chemotherapy agents can modulate procoagulant pathways, we measured the effects of platinum-based chemotherapy on cell surface TF activity of HUVECs, A549 cells, and monocytes. Cells were treated with single agent and combination chemotherapy, and cell surface TF activity was assessed by FXa generation. We observed a significant increase in TF activity on HUVECs treated with 1 uM and 30 uM cisplatin alone, 10 uM gemcitabine alone, and cisplatin/gemcitabine combination therapy (1 uM and 30 uM, Figure 3A). We also observed significant increases in TF activity on HUVECs treated with 10 uM and 30 uM paclitaxel and carboplatin/paclitaxel combination therapy (1-30 uM, Figure 3B).

Furthermore, we also observed chemotherapy-induced procoagulant effects on A549 cells and monocytes. TF activity was significantly increased on A549 cells treated with 10-30 uM cisplatin/gemcitabine combination as well as with high dose (30 uM) cisplatin as a single agent (Figure 4A). Treatment of A549 cells with carboplatin alone did not result in significant increases of TF activity at any dose (1-30 uM), whereas treatment of A549 cells with paclitaxel alone (10 uM and 30 uM) and all doses of carboplatin/paclitaxel combination resulted in significant increases in TF activity (Figure 4B). Interestingly, we observed increased cell surface TF activity on monocytes treated with all doses of cisplatin and gemcitabine as single agent treatments or in combination (Figure 5A). Cell surface FXa generation was increased on monocytes treated with carboplatin ($p = 0.08$) and paclitaxel ($p = 0.052$) (Figure 5B, $n = 4$).

**Figure 3. Effects of single agent and combination chemotherapy on HUVEC cell surface TF activity.**

HUVEC were treated with single agent and combination chemotherapy for 24 hours. FXa generation was measured on HUVEC treated with (A) cisplatin and gemcitabine and (B) carboplatin and paclitaxel as single agent only or combination treatments. Data represents mean ± SEM of at least 3 individual experiments. Values are expressed as mean $\pm$ SE. Significant difference compared to untreated condition is denoted by: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

(A)


FXa Generated (pM/10^4 Cells)
Untreated
Cisplatin
Gemcitabine
Cisplatin + Gemcitabine
1 uM
10 uM
30 uM
Drug Concentration

(B)

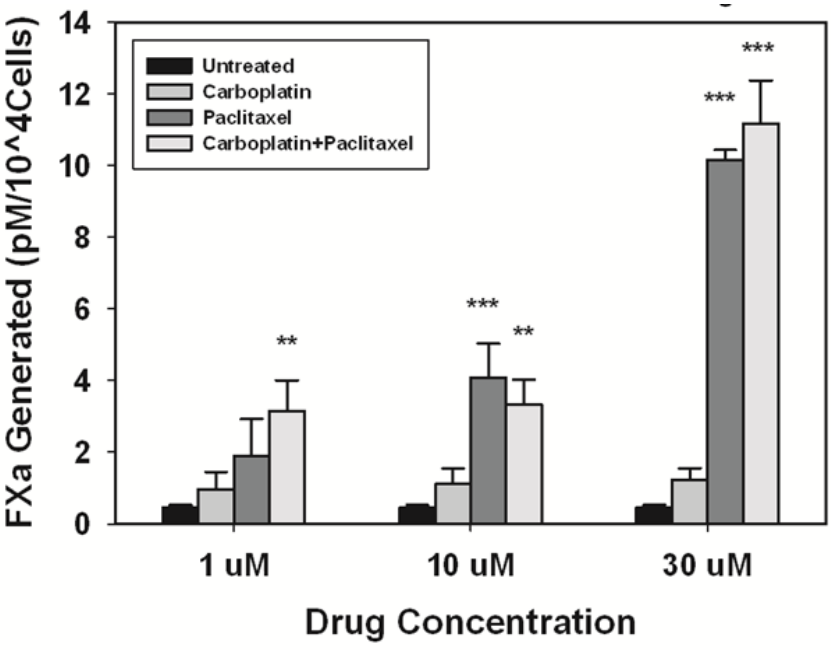
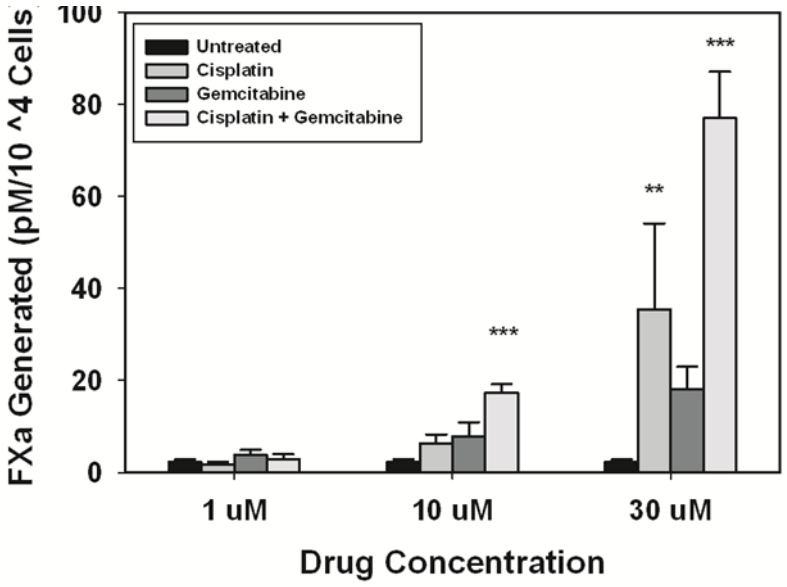

FXa Generated (pM/10^4Cells)
Untreated
Carboplatin
Paclitaxel
Carboplatin+Paclitaxel
1 uM
10 uM
30 uM
Drug Concentration

**Figure 4. Effects of single agent and combination chemotherapy on A549 cell surface TF activity.**

A549 cells were treated with single agent and combination chemotherapy for 24 hours. FXa generation was measured on A549 cells treated with (A) cisplatin and gemcitabine and (B) carboplatin and paclitaxel as single agent only or combination treatments. Data represents mean ± SEM of at least 3 individual experiments. Values are expressed as mean $\pm$ SE. Significant difference compared to untreated condition is denoted by: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

(A)


Untreated
Cisplatin
Gemcitabine
Cisplatin + Gemcitabine
FXa Generated (pM/10 ^4 Cells)
100
80
60
40
20
0
***
**
***
1 uM
10 uM
30 uM
Drug Concentration

(B)

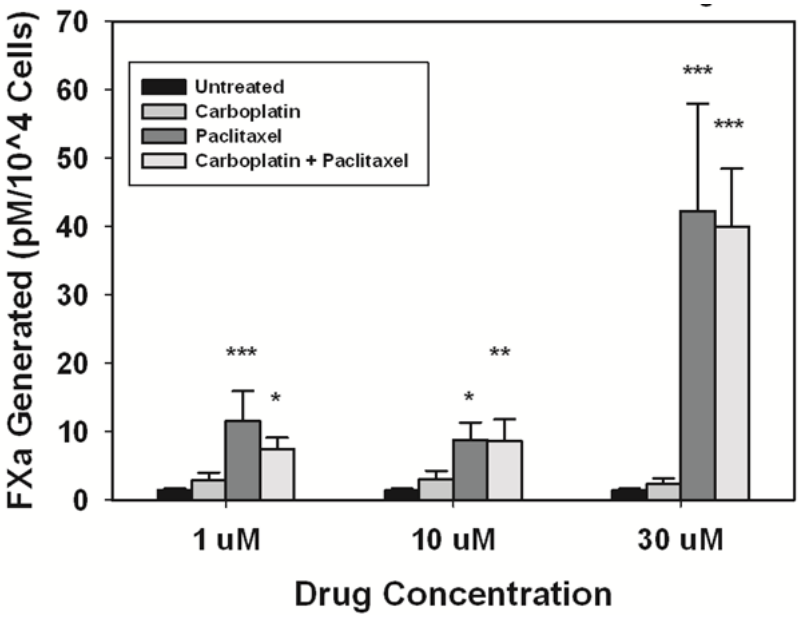
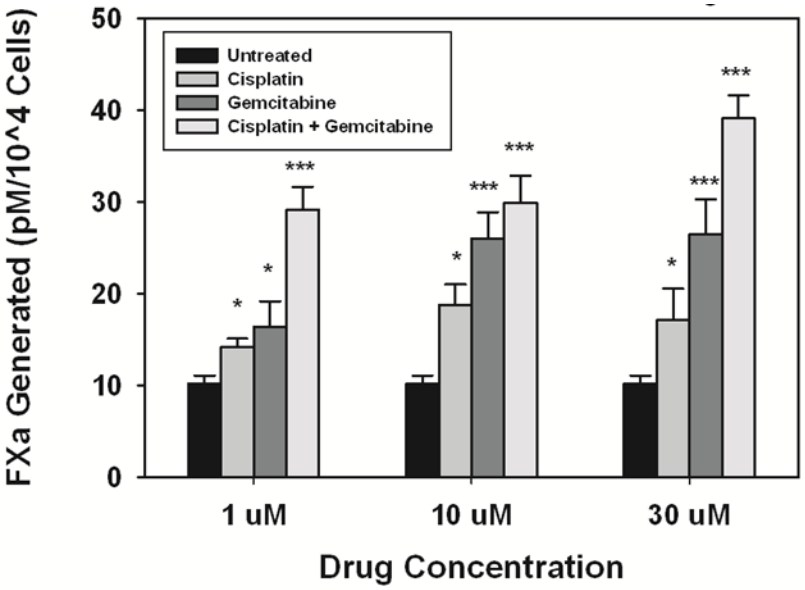

Untreated
Carboplatin
Paclitaxel
Carboplatin + Paclitaxel
FXa Generated (pM/10^4 Cells)
70
60
50
40
30
20
10
0
***
*
*
**
***
***
1 uM
10 uM
30 uM
Drug Concentration

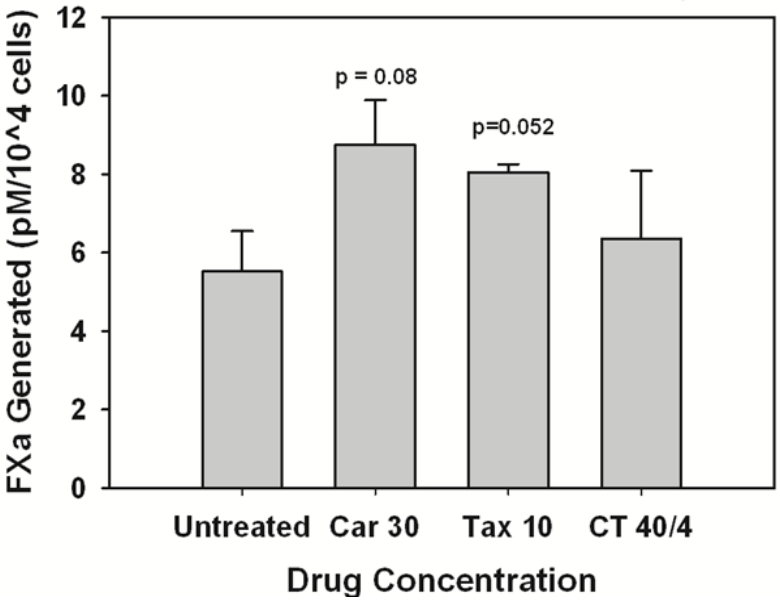
**Figure 5. Effects of single agent and combination chemotherapy on monocyte cell surface TF activity.**

Monocytes were treated with single agent and combination chemotherapy for 24 hours. FXa generation was measured on monocytess treated with (A) cisplatin and gemcitabine and (B) carboplatin and paclitaxel as single agent only or combination treatments. Data represents mean ± SEM of at least 3 individual experiments. Values are expressed as mean $\pm$ SE. Significant difference compared to untreated condition is denoted by: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

(A)


FXa Generated (pM/10^4 Cells)
50
40
30
20
10
0
Untreated
Cisplatin
Gemcitabine
Cisplatin + Gemcitabine
*
*
***
*
***
***
*
***
***
1 uM
10 uM
30 uM
Drug Concentration

(B)


FXa Generated (pM/10^4 cells)
12
10
8
6
4
2
0
p = 0.08
p=0.052
Untreated
Car 30
Tax 10
CT 40/4
Drug Concentration

In order to determine if chemotherapy-induced cell surface FXa generation was TF dependent, we repeated the FXa generation experiments as described in methods using a buffer containing FX only or FX and FVIIa, an essential component of the extrinsic tenase. FXa generation on HUVEC and A549 cells was diminished below FXa generation levels observed in the untreated condition when performed using the buffer containing FX only (Figure 6). We also examined cell surface FXa generation in the presence of a TF inhibiting antibody (HTF-1). A549 cells and monocytes were cultured and treated with combination therapy as described in methods. Prior to initiation of FXa generation assay, the cells were pre-incubated with 10 ug/ml of HTF-1 for 30 minutes at room temperature. Our results demonstrate a complete ablation of FXa generation on A549 cells (Figure 7A) and monocytes treated with cisplatin/gemcitabine as well as with carboplatin/ paclitaxel when performed in the presence of a TF inhibiting antibody (Figure 7B).

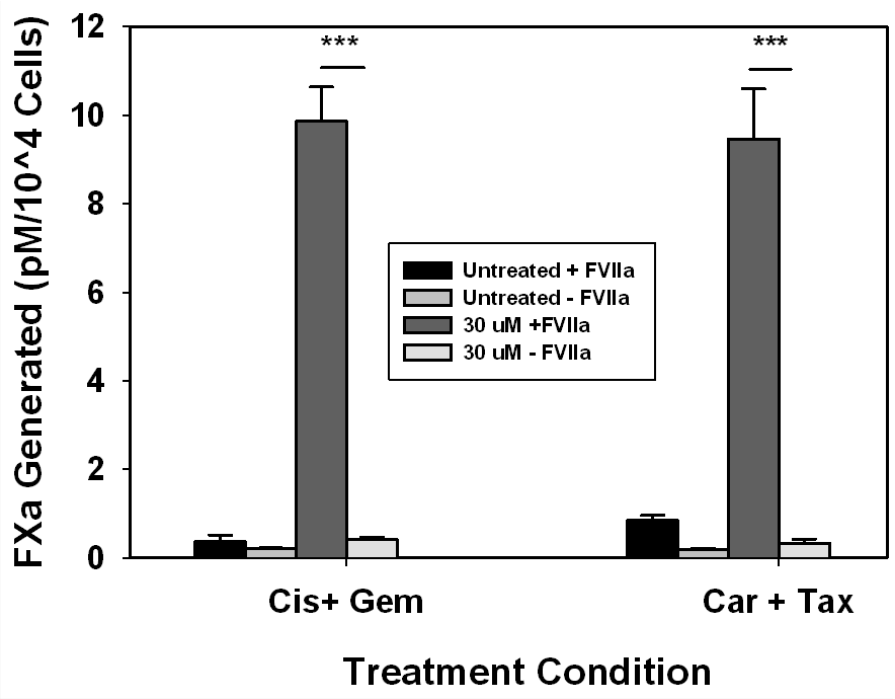
### 3.2 Effects of platinum-based chemotherapy on TF antigen levels

The increase in TF activity observed on chemotherapy treated HUVEC, A549 cells, and monocytes could be attributed to increased levels of cell surface TF antigen or increased procoagulant activity levels of existing antigen (TF decryption). We observed no changes in cell surface levels of TF antigen on HUVEC and A549 cells under all chemotherapy treatment conditions (Figure 8A). In contrast to HUVEC and A549 cells, we observed a significant increase in cell surface TF antigen level on monocytes treated with high dose cisplatin only as well as high dose cisplatin/gemcitabine combination

**Figure 6. Effects of combination chemotherapy on cell surface TF activity.**

Cells were treated with combination chemotherapy for 24 hours. FXa generation was measured on chemotherapy (A) HUVEC and (B) A549 cells in the presence or absence of FVIIa. Data represents mean ± SEM of at least 3 individual experiments. Values are expressed as mean $\pm$ SE. Significant difference compared to untreated condition is denoted by: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

(A)


FXa Generated (pM/10^4 Cells)
12
10
8
6
4
2
0
***
***
Untreated + FVIIa
Untreated - FVIIa
30 uM +FVIIa
30 uM - FVIIa
Cis+ Gem
Car + Tax
Treatment Condition

(B)

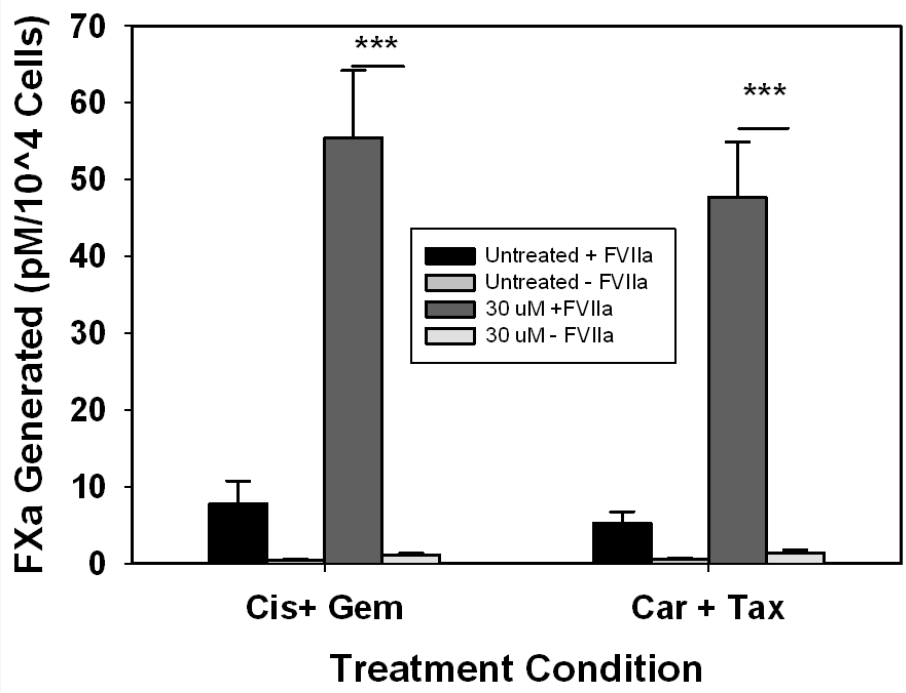
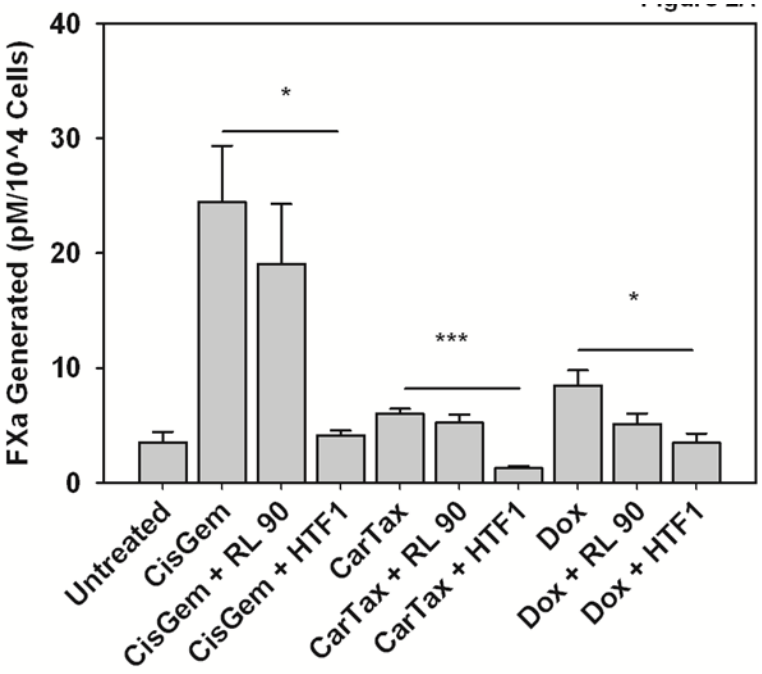

FXa Generated (pM/10^4 Cells)
70
60
50
40
30
20
10
0
***
***
Untreated + FVIIa
Untreated - FVIIa
30 uM +FVIIa
30 uM - FVIIa
Cis+ Gem
Car + Tax
Treatment Condition

**Figure 7. Effects of combination chemotherapy on cell surface TF activity.**

Cells were treated with combination chemotherapy for 24 hours. Cell surface TF activity was measured on combination **A)** A549 cells in the presence of HTF-1 and RL90 (TF and phosphatidylserine inhibiting antibodies respectively) and **B)** monocytes in the presence of HTF-1 and Annexin-V. Values are expressed as mean $\pm$ SE. Significant differences between treatment and treatment + HTF-1 conditions are denoted by: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

(A)


FXa Generated (pM/10^4 Cells)
40
30
20
10
0
*
***
*
Untreated
CisGem
CisGem + RL 90
CisGem + HTF1
CarTax
CarTax + RL 90
CarTax + HTF1
Dox
Dox + RL 90
Dox + HTF1

(B)

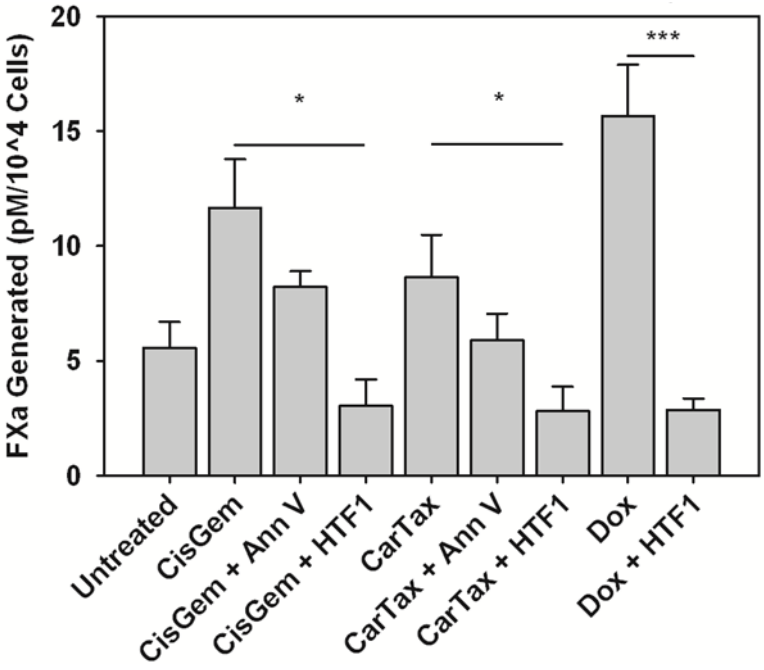
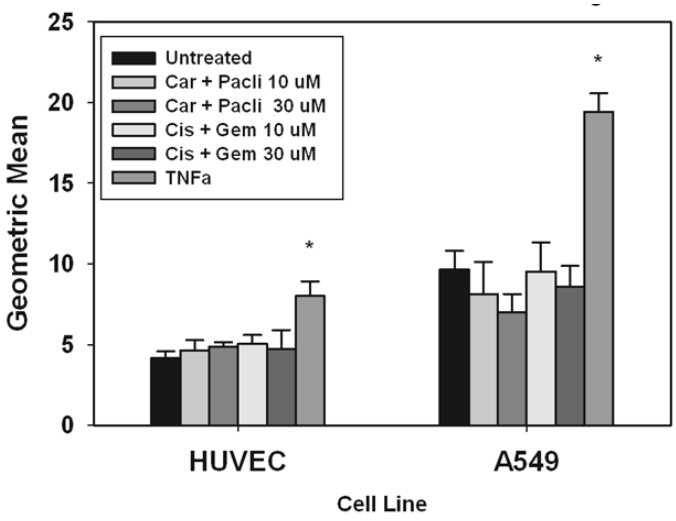

FXa Generated (pM/10^4 Cells)
20
15
10
5
0
*
*
***
Untreated
CisGem
CisGem + Ann V
CisGem + HTF1
CarTax
CarTax + Ann V
CarTax + HTF1
Dox
Dox + HTF1

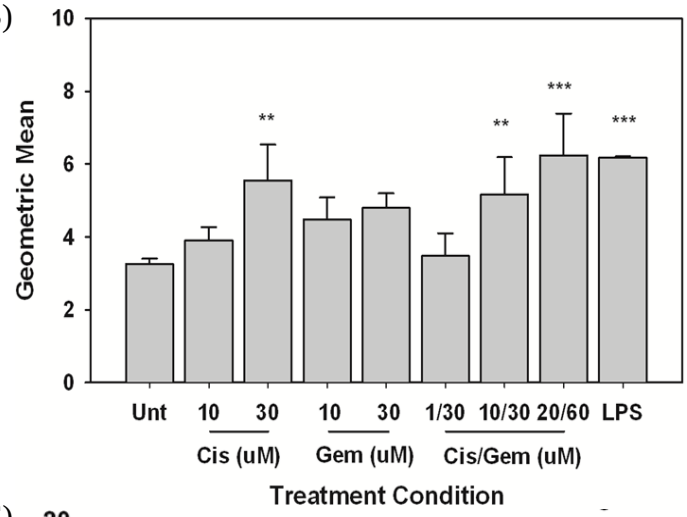
**Figure 8. Effects of chemotherapy on cell surface TF antigen levels.**

Cell surface TF antigen levels on **A)** HUVEC and A549 cells treated with carboplatin/paclitaxel as well as cisplatin/gemcitabine combination therapies, **B)** monocytes treated with cisplatin and gemcitabine and **C)** monocytes treated with carboplatin and paclitaxel. Values are expressed as mean $\pm$ SE. Significant difference compared to untreated condition is denoted by: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

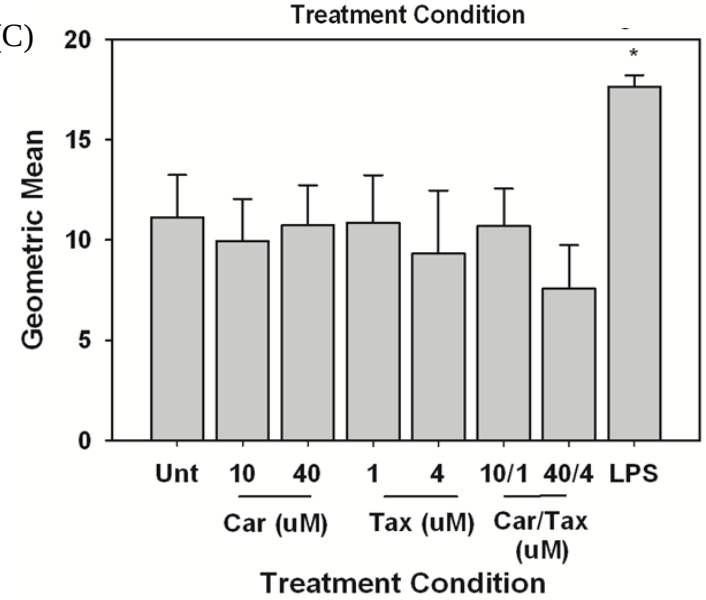
(A)


Untreated
Car + Pacli 10 uM
Car + Pacli 30 uM
Cis + Gem 10 uM
Cis + Gem 30 uM
TNFa
Geometric Mean
25
20
15
10
5
0
*
*
HUVEC
A549
Cell Line

(B)


Geometric Mean
10
8
6
4
2
0
**
**
***
***
Unt
10
30
10
30
1/30
10/30
20/60
LPS
Cis (uM)
Gem (uM)
Cis/Gem (uM)
Treatment Condition

(C)


Geometric Mean
20
15
10
5
0
*
Unt
10
40
1
4
10/1
40/4
LPS
Car (uM)
Tax (uM)
Car/Tax (uM)
Treatment Condition

treatment (Figure 8B) while no change in cell surface TF was observed on monocytes treated with carboplatin and paclitaxel (Figure 8C).

**3.3 Effects of platinum-based chemotherapy on phosphatidylserine exposure**

To determine the effect of chemotherapy on phosphatidylserine exposure, we measured annexin-V binding to chemotherapy treated cells using flow cytometry. Annexin V is a $Ca^{2+}$ dependent phospholipid binding protein that binds to cell surface phosphatidylserine with high affinity (Vermes et al. 1995). We observed a dose-dependent increase in phosphatidylserine exposure on HUVEC treated with cisplatin/gemcitabine combination at 10 uM and higher concentrations (Figure 9A). We did not observe a significant increase in Annexin V binding on HUVEC treated with the carboplatin/paclitaxel combination therapy. However, higher concentration of carboplatin/paclitaxel combination therapy (30 uM) resulted in increased phosphatidylserine exposure that was approaching significance ($p=0.075$) (Figure 9B). In contrast to the effect seen on HUVEC, treatment of A549 cells with cisplatin and gemcitabine or carboplatin/paclitaxel combination therapy (Figure 10) did not result in significant increases in phosphatidylserine exposure at any treatment concentration.

In monocytes, treatment with 10 uM cisplatin resulted in significant phosphatidylserine exposure (Figure 11A). Monocytes treated with high-dose gemcitabine as well as cisplatin/gemcitabine combination therapy expressed an approximate 2-fold increase in phosphatidylserine exposure ($p = 0.08$) (Figure 11A). Interestingly, while monocytes treated with carboplatin did not express increased levels of

phosphatidylserine exposure, addition of high-dose paclitaxel (4 uM) to carboplatin resulted in a significant reduction of phosphatidylserine exposure (Figure 11B).

**3.4 Mechanisms of chemotherapy induced TF decryption**

Next, we examined the ability of cell surface phosphatidylserine on chemotherapy-treated HUVEC and monocytes to facilitate TF decryption. Phosphatidylserine was blocked using purified recombinant annexin V at 25µg/mL for 15 minutes prior to performing the TF activity assay. While cisplatin/gemcitabine combination treatment significantly increased cell surface TF activity on HUVEC, TF activity was not affected by annexin V binding (Figure 12). In contrast to HUVEC, inhibition of phosphatidylserine on chemotherapy treated monocytes resulted in a modest attenuation of cell surface FXa generation (Figure 7B).

Protein disulphide isomerase (PDI) has been postulated to contribute to TF decryption through the Cys186-Cys209 disulphide bond formation (Pendurthi et al. 2007). To determine if PDI activity was contributing to increased TF activity on A549 cells as a result of increased TF decryption, TF activity was measured on chemotherapy treated A549 cells that were incubated with the PDI inhibitory antibody (RL-90) prior to platinum-based chemotherapy treatment. Our results show that pretreatment of A549 cells with the PDI inhibitory antibody did not attenuate the cisplatin/gemcitabine or carboplatin/paclitaxel combination therapy induced TF activity.

**3.5 Procoagulant effects of lung cancer chemotherapy agents *in vivo*.**

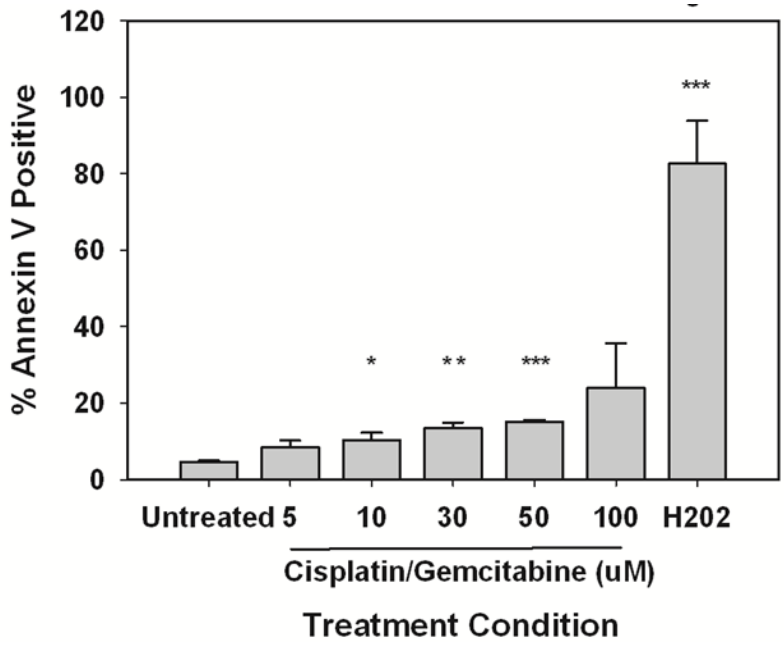
To assess the relevance of our *in vitro* findings *in vivo*, we investigated the procoagulant effects of lung cancer chemotherapy and anti-angiogenesis therapy in a

mouse model. We injected healthy Balb/C mice with lung cancer chemotherapy ± Avastin (bevacizumab) or saline as negative control on three consecutive days and measured the resulting TAT levels. Our results demonstrate a modest upregulation of TAT levels in mice treated with paclitaxel and carboplatin as single agent chemotherapy (Figure12). The addition of an anti-angiogenic agent to single agent therapy did not correspond to an increase in TAT levels.

**Figure 9. Effect of chemotherapy on HUVEC cell surface phosphatidylserine exposure.**

Phosphatidylserine exposure levels on HUVEC treated with (A) cisplatin/gemcitabine combination or (B) carboplatin/paclitaxel combination chemotherapy. Values are expressed as mean ± SE. Significant difference compared to untreated condition is denoted by: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

(A)


120
100
80
60
40
20
0
% Annexin V Positive
***
*
**
***
Untreated
5
10
30
50
100
H202
Cisplatin/Gemcitabine (uM)
Treatment Condition

(B)

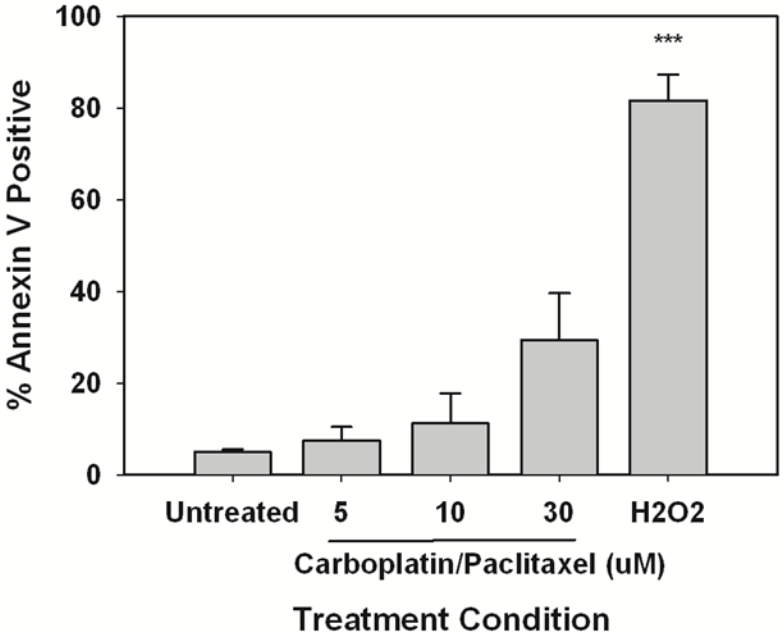

100
80
60
40
20
0
% Annexin V Positive
***
Untreated
5
10
30
H2O2
Carboplatin/Paclitaxel (uM)
Treatment Condition

**Figure 10. Effect of chemotherapy on A549 cell surface phosphatidylserine exposure.**

Phosphatidylserine exposure levels on A549 cell treated with cisplatin/gemcitabine or carboplatin/paclitaxel combination therapies. Values are expressed as mean $\pm$ SE. Significant difference compared to untreated condition is denoted by: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

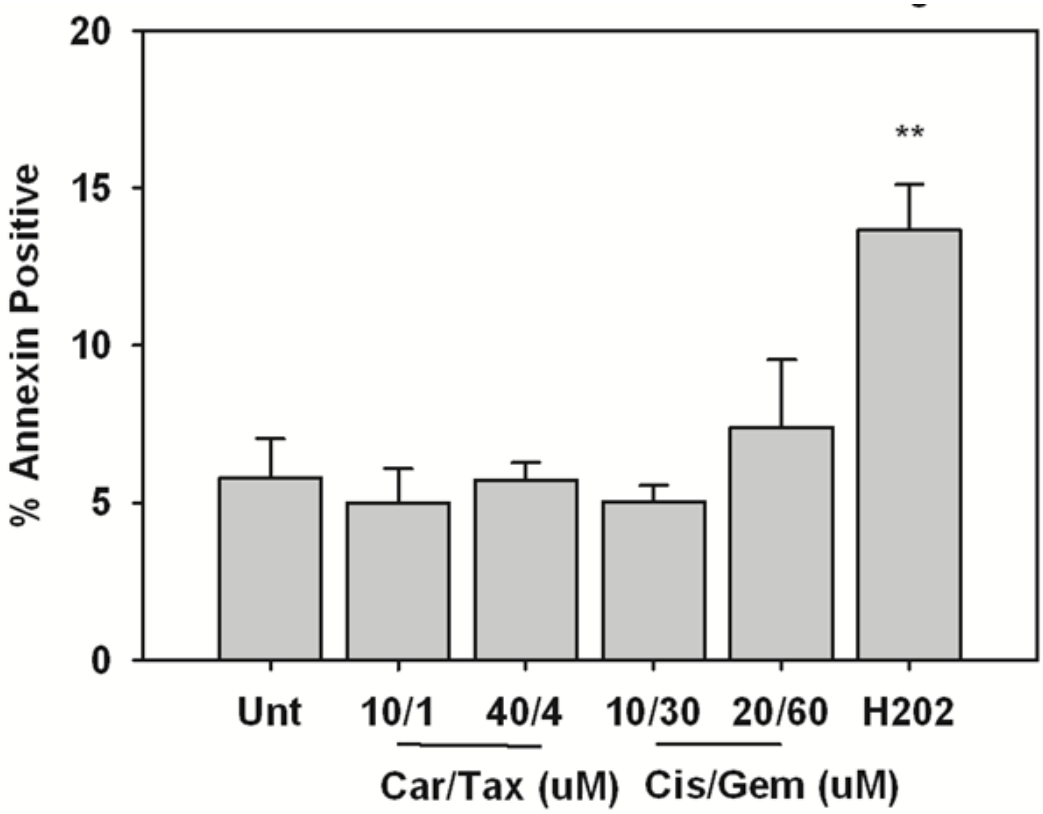
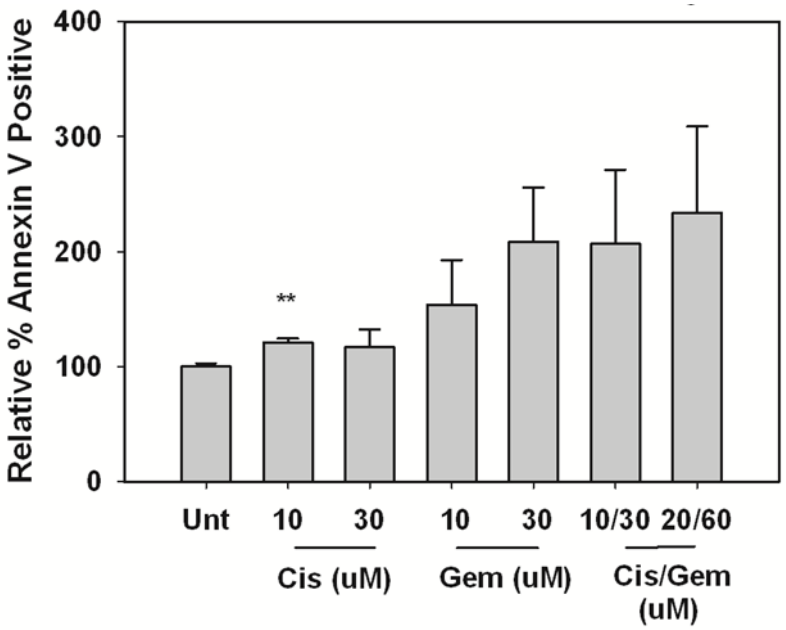

20
15
10
5
0
% Annexin Positive
**
Unt
10/1
40/4
10/30
20/60
H202
Car/Tax (uM)
Cis/Gem (uM)

**Figure 11. Effect of chemotherapy on monocyte cell surface phosphatidylserine exposure.**

Phosphatidylserine exposure levels on HUVEC treated with (A) cisplatin/gemcitabine or (B) carboplatin/paclitaxel combination therapies. Values are expressed as mean $\pm$ SE. Significant difference compared to untreated condition is denoted by: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

(A)


Relative % Annexin V Positive
400
300
200
100
0
**
Unt
10
30
10
30
10/30
20/60
Cis (uM)
Gem (uM)
Cis/Gem (uM)

(B)

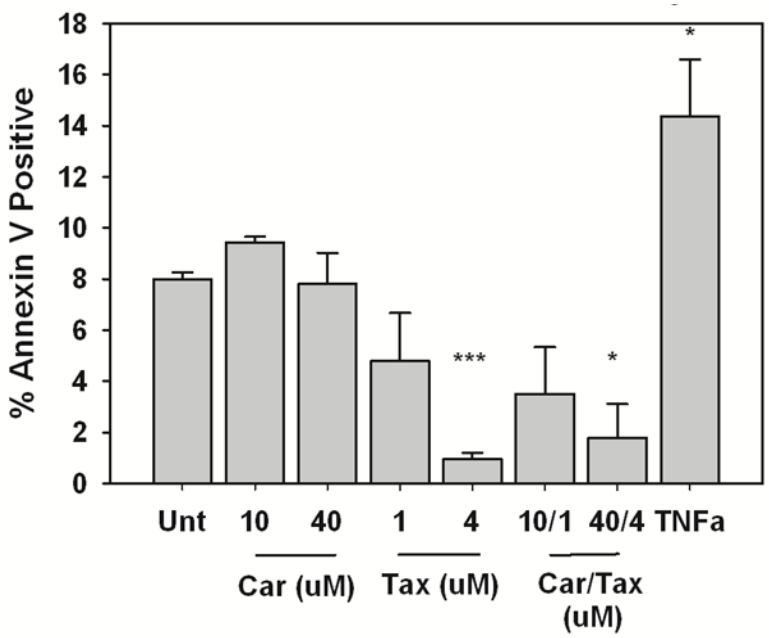

% Annexin V Positive
18
16
14
12
10
8
6
4
2
0
***
*
*
Unt
10
40
1
4
10/1
40/4
TNFa
Car (uM)
Tax (uM)
Car/Tax (uM)

**Figure 12. Effect of phosphatidylserine on TF activity of chemotherapy treated HUVEC.**

Cell surface TF activity on single agent and combination chemotherapy treated HUVEC in the presence or absence of Annexin-V. Values are expressed as mean ± SE. Significant compared to the untreated condition is denoted by: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

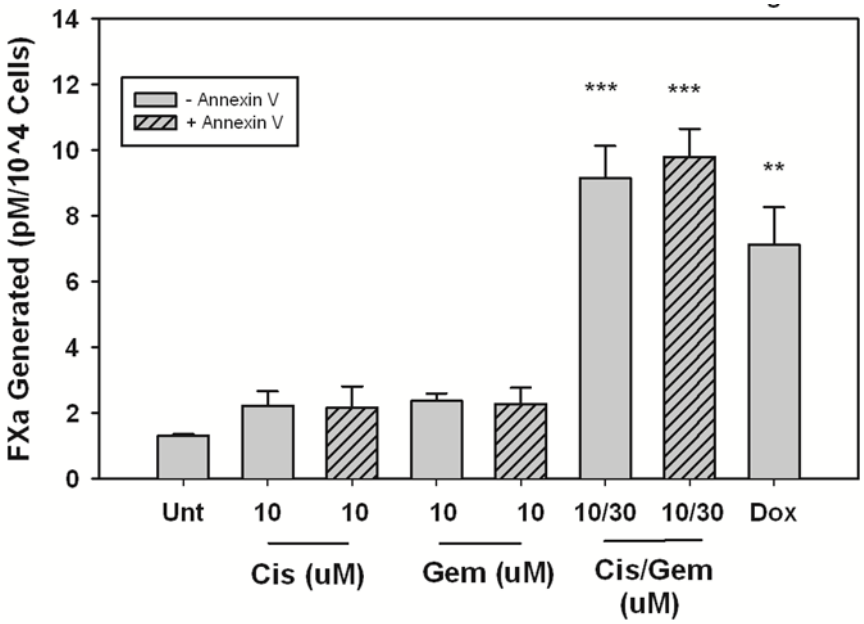
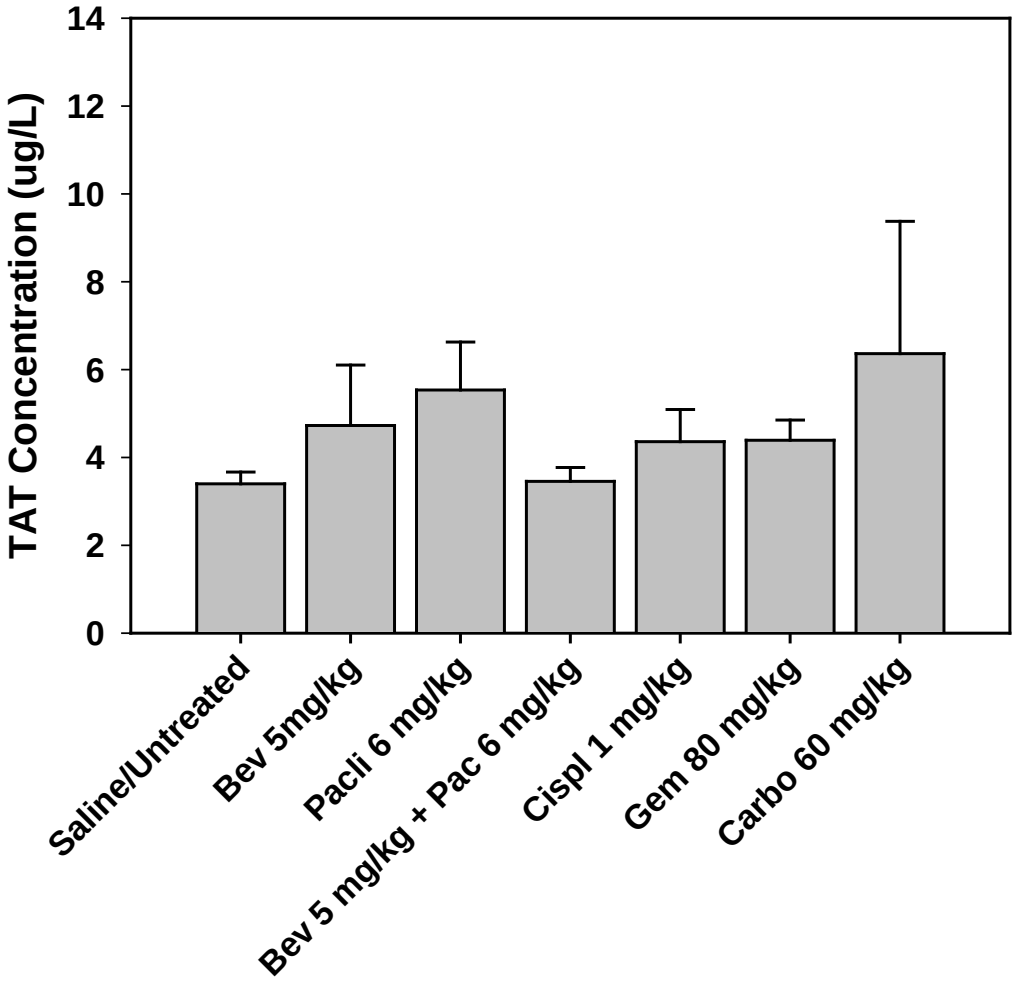

FXa Generated (pM/10^4 Cells)
14
12
10
8
6
4
2
0
- Annexin V
+ Annexin V
***
***
**
Unt
10
10
10
10
10/30
10/30
Dox
Cis (uM)
Gem (uM)
Cis/Gem (uM)

**Figure 13. Effects of anti-lung cancer therapy on plasma TAT complex concentrations in healthy mice.**

Healthy Balb/C mice were injected intraperitoneally with anti-lung cancer agents on days 1, 2 and 3. Blood collected was performed on day 4 (24 hours post final injection). Cell surface TF activity on single agent and combination chemotherapy treated HUVEC in the presence or absence of Annexin-V. Values are expressed as mean $\pm$ SE.


TAT Concentration (ug/L)
14
12
10
8
6
4
2
0
Saline/Untreated
Bev 5mg/kg
Pacli 6 mg/kg
Bev 5 mg/kg + Pac 6 mg/kg
Cispl 1 mg/kg
Gem 80 mg/kg
Carbo 60 mg/kg

**4.0 Discussion:**

Thrombosis is a common complication in cancer patients undergoing chemotherapy and anti-angiogenesis therapy (Goodnough et al. 1984; Khorana et al. 2005; Tesselaar and Osanto 2007). While chemotherapeutic and anti-angiogenic agents elicit anti-tumor effects through distinct mechanisms, both therapies increase the risk of thrombosis in cancer patients (Johnson et al. 2004). Chemotherapeutic agents elicit their anti-tumor effects through mitotic arrest and induction of apoptosis of rapidly dividing cells, whereas anti-angiogenic agents elicit anti-tumor effects through prevention of vasculature formation to areas of hypoxia through inhibition of VEGF and/or VEGFR signaling pathways (Bikfalvi and Bicknell 2002).

Although the association between the use of anti-lung cancer therapeutic agents and thrombosis is well recognized, the mechanism(s) by which these agents can increase the risk for thrombosis remain largely unknown. A study of patients with stage IIIB and IV non small cell lung cancer receiving combination chemotherapy with carboplatin and paclitaxel demonstrated that thrombin-antithrombin complex formation was elevated between 1 and 48 hours post-chemotherapy (Weitz et al. 2002). Our results provide experimental evidence and insight into the potential mechanisms by which lung cancer chemotherapy agents can modulate procoagulant (thrombin generating) pathways.

Our studies demonstrate an increase in cell surface TF activity as measured by FXa generation on HUVEC treated with cisplatin and gemcitabine. This is consistent with studies that have demonstrated increased TF activity on HUVEC treated with these chemotherapy agents (Ma et al. 2005). However, in contrast to Ma et al. (2005) we did

not observe an increase in TF antigen levels on HUVEC treated with the cisplatin/gemcitabine combination. Our findings of increased TF activity on paclitaxel treated HUVEC are also contradictory to those of Napoleone et. al (2009) who reported a decrease in TF activity on paclitaxel treated endothelial cells using a one stage clotting assay in human plasma (Napoleone et al. 1997). We hypothesize that the conflicting results may reflect the different experimental methodologies to measure TF activity and antigen levels. In our studies, flow cytometry was used to measure cell surface TF expression on HUVEC, monocytes, and A549 cells, whereas Ma et al. (2005) utilized an ELISA (American Diagnostica) technique. Our flow cytometry results were confirmed by a TF antigen ELISA (R&D systems) performed on chemotherapy-treated A549 cells. We also extended previous in vitro studies by investigating the procoagulant effects of carboplatin alone and in combination with paclitaxel on endothelial cells.

This study is the first to report effects of lung cancer chemotherapeutic agents on monocytes and A549 cell hemostatic pathways as a result of single agent as well as platinum-based combination chemotherapy treatment. It is interesting to note that 30 uM cisplatin/gemcitabine treatment resulted in approximately 2- and 6.5-fold higher FXa generation on A549 cells as compared with monocytes and HUVEC respectively. Moreover, FXa generation was approximately 4-fold higher on A549 cells treated with 30 uM carboplatin/paclitaxel combination therapy relative to HUVEC and monocytes. This could be attributed to higher baseline levels of cell surface TF antigen on tumor cell lines for TF-FVIIa complex formation. TF antigen expression has been reported to play a role in tumor metastasis. A study of surgically resected NSCLC tumors found a high

correlation between tumor metastasis and TF antigen expression demonstrating that high basal levels of TF antigen expression could have implications in tumor progression (Sawada et al. 1999) and contribute to increased procoagulant activity associated with the presence of a malignancy.

Our studies demonstrate increased cell surface TF activity on chemotherapy treated HUVEC, monocytes, and A549 cells. However, only chemotherapy-treated monocytes express increased levels of cell surface TF antigen. Although lung cancer chemotherapy elicits procoagulant effects on all three cell lines studied, only treatment of monocytes with cisplatin and gemcitabine as single agent only or in combination results in an increase in both TF procoagulant activity and antigen levels. This suggests that the cisplatin/gemcitabine chemotherapeutic agent combination exerts the greatest procoagulant effect on monocytes. Although this does not result in the highest levels of FXa generation, the significant increase in FXa generated at all treatment concentrations (1-30 uM) of cisplatin and gemcitabine as single agent only and in combination demonstrates high sensitivity of monocytes to these anti-lung cancer agents.

Interestingly, while we see a significant increase in TF activity on monocytes treated with lower concentrations (1 uM and 10 uM) of cisplatin and gemcitabine alone and in combination, TF antigen levels remain unchanged at those treatment concentrations and only increase at high dose (30 uM ) cisplatin only and high dose combination therapy. Since the increase in TF antigen occurs in conditions containing >30 uM cisplatin, TF antigen increase on monocytes appears to be due to the high concentration of cisplatin.

The formation of the extrinsic tenase complex upon TF exposure increases the proteolytic activity of FVIIa (a serine protease) by approximately 3 orders of magnitude, which in turn is able to activate FIX and FX (Lawson et al. 1992; Dahlback 2008). Cancer procoagulant (CP) is a cysteine protease that is capable of directly activating FX independently of the extrinsic tenase complex (Gordon et al. 1975). Increased CP activity has been reported in patients with lung, breast, and colorectal cancers and studied as a potential early cancer detection marker (Kozwich et al. 1994; Rucinska et al. 1997).

Since we used FXa generation to monitor TF activity, we wanted to determine if any of the generated FXa on A549 cell surface was attributable to CP activity. We studied cell surface TF activity on untreated and chemotherapy treated A549 cells in the presence or absence of FVIIa. In the absence of FVIIa, FXa generation can be attributed to CP activity. Our experiments demonstrate negligible FXa generation in FVIIa-deficient buffer, suggesting that FX activation on A549 cells was in fact FVIIa-dependent, which in turn requires the formation of the TF-FVIIa complex. Moreover, the complete ablation of FXa generation on A549 cells and monocytes in the presence of a TF neutralizing antibody confirms that our findings of chemotherapy induced TF activity on A549 cells is TF-dependent.

Taken together, data obtained from these experiments suggest that the increase in TF activity without a corresponding increase of cell surface TF antigen on HUVEC, A549 cells, and monocytes treated with lower dose chemotherapy is likely due to TF decryption. However, due to corresponding increased levels of TF activity and TF antigen levels on monocytes treated with high dose cisplatin only and high dose

cisplatin/gemcitabine combination, the observed increase in cell surface TF activity can at least in part be attributed to changes in TF antigen levels.

Numerous mechanisms of TF decryption have been postulated throughout the literature including increased phosphatidylserine exposure and PDI activity (Zwaal and Schroit 1997; Lentz 2003; Pendurthi et al. 2007). The association between increased phosphatidylserine exposure and procoagulant activity is well recognized (Zwaal and Schroit 1997; Lentz 2003). In addition, inhibition of phosphatidylserine has been shown to attenuate TF activity and thrombin generation on chemotherapy treated cells (Swystun et al. 2009)

In our studies, the procoagulant effects of two platinum-based chemotherapy regimens on HUVEC were investigated. Although increased TF procoagulant activity was observed on HUVEC treated with either platinum-based combination therapy, only HUVEC treated with cisplatin/gemcitabine combination expressed increased levels of phosphatidylserine exposure. Furthermore, inhibition of phosphatidylserine on these cells did not attenuate the increase in TF activity, suggesting that the chemotherapy-induced increase in TF activity seen on HUVEC is due to TF decryption that is not phosphatidylserine mediated. As phosphatidylserine exposure was not increased on chemotherapy treated A549 cells, we examined the role of PDI on TF activity of chemotherapy treated A549 cells next. Our results indicate that inhibition of PDI in cisplatin-gemcitabine and carboplatin-paclitaxel treated A549 cells does not affect cell surface TF activity.

Interestingly, we witnessed a significant reduction in phosphatidylserine exposure on monocytes treated with paclitaxel alone and in combination with carboplatin compared to the untreated condition, whereas treatment of monocytes with carboplatin alone did not modulate phosphatidylserine levels. Sequestering of phosphatidylserine to the inner or outer leaflet of the cell is a result of a dynamic function between flippase and floppase to translocate phosphatidylserine to the inner and outer leaflet of the cell membrane, respectively (Daleke 2003; Zwaal et al. 2005). Therefore, the observed reduction in phosphatidylserine on monocytes is unique to the mechanism of action of paclitaxel. Paclitaxel differs in mechanism of action from the other three studied lung cancer chemotherapy agents in that it does not bind DNA, but rather stabilizes the microtubule environment of the cell. We hypothesize that paclitaxel binding to β-tubulin and/or stabilization of the microtubule environment may have indirect effects on the function of phospholipid translocases (flippase and floppase) resulting in decreased translocation of phosphatidylserine to the outer leaflet of the cell membrane.

Plasma concentration of thrombin-antithrombin (TAT) complexes are often used as a marker of thrombin inactivation. To asses if our *in vitro* findings were relevant *in* vivo, we tested the effect of lung cancer chemotherapy agents on the formation of TAT complex in healthy Balb/C mice. A recent study has also demonstrated that cellular uptake of paclitaxel is increased when used in conjunction with bevacizumab (Yanagisawa et al. 2010). Furthermore, the addition of bevacizumab to cisplatin/paclitaxel combination therapy resulted in an increased incidence of VTE in lung cancer patients (Cohen et al. 2007). Therefore we investigated the procoagulant

effects of bevacizumab administered alone and in combination with paclitaxel. Surprisingly, no significant elevation in plasma TAT complex levels was observed. It is important to note that the half-life of TAT complex is approximately 3 minutes *in vivo* (Shifman and Pizzo 1982). Since blood was collected 24 hours post final injection, the TAT complex levels could have returned to baseline during this time period. Furthermore, with the exception of Avastin and paclitaxel adjuvant treatment, we investigated the *in vivo* procoagulant effects of single agent treatment only. We also hypothesize that implementing combination therapy in the presence or absence of bevacizumab would further elevate TAT plasma levels due to increased incidence rates of thrombosis associated with combination therapy use in the clinical setting.

Collectively, our studies suggest that lung cancer chemotherapy agents can modulate hemostatic pathways through increased tissue factor activity on healthy host tissues as well as malignant non small cell lung cancer cells. This suggests that the administration of platinum-based chemotherapy to lung cancer can induce activation of coagulation localized to the site of the tumour as well as systemically due to activation of monocytes and endothelial cells. In addition, previous studies have suggested that lung cancer chemotherapy is also associated with increased levels of procoagulant microparticles (Lechner et al. 2007), which may further enhance this procoagulant response. Our studies elucidate mechanisms by which chemotherapy agents can increase thrombin generation in lung cancer patients receiving chemotherapy and may prove to be useful in development of prophylactic anticoagulant strategies for cancer patients receiving chemotherapy.

## 5.0 Future Directions

### 5.1 Effects of lung cancer chemotherapy agents on anti-coagulant pathways

Previous studies from our lab have demonstrated a reduction in cell surface EPCR and an increase in TM levels on endothelial cells treated with breast cancer chemotherapy agents (Mukherjee et al. ; Woodley-Cook et al. 2006). Also, it has been demonstrated that treatment of HUVEC with cisplatin and gemcitabine as single agent and combination therapy resulted in decreased TFPI activity, most likely due to corresponding decreases in TFPI expression levels (Ma et al. 2005).

Although the effects of some lung cancer chemotherapy agents on procoagulant pathways have been demonstrated, it remains unknown whether lung cancer chemotherapy also downregulates anticoagulant pathways. Effects of single agent only and combination chemotherapy on anticoagulant pathways could be studied by measuring expression levels of EPCR, TM, and TFPI on chemotherapy treated HUVEC. These experiments would help us determine if increased incidence rates of thrombosis in lung cancer patients receiving chemotherapy are a result of simultaneous modulation of both pro- and anticoagulant pathways.

### 5.2 Effect of platinum-based combination chemotherapy and anti-angiogenesis therapy on pro- and anticoagulant pathways *in vivo*.

Cancer patients undergoing chemotherapy have been reported to have increased TAT plasma concentration shortly after initiation of chemotherapy (Weitz et al. 2002). To assess the effects of lung cancer chemotherapy agents on thrombin generation pathways

in the absence of cancer, plasma TAT complex concentration can be measured in healthy mice. Establishing levels of plasma TAT complex in healthy mice 1-48 hours pos treatment with single agent and combination chemotherapy in the presence or absence of anti-angiogenesis agents will allow us to identify the procoagulant effects of specific therapeutic agents.

It is also important to determine if TF antigen levels remain constant post chemotherapy treatment as was observed in our *in vitro* studies. Immunohistochemical methods can be employed to examine TF antigen levels in livers of mice treated with single agent, combination chemotherapy or chemotherapy in combination with antiangiogenesis therapy. Specimens collected from treated animals can be prepared for frozen sectioning as previously described (Saito et al. 2009), and labeled using a primary rabbit anti-mouse tissue factor antibody (American Diagnostica). These results would demonstrate whether chemotherapy exerts procoagulant effects *in vivo* through TF activation (decryption) and/or upregulation of antigen levels.

Furthermore, no studies have been published to date on anti-lung cancer therapy effects on anti-coagulant pathways *in vivo.* Identification of the mechanism by which anti-lung cancer therapies mkodulate anti-coagulant pathways *in vivo* (protein C, APC), may provide insight into strategies to customize antithrombotic prophylaxis tailored to the specific chemotherapy regiment that the patient receives.

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