

## ***Inhibitory Effect of Paclitaxel and Rapamycin Individual and Dual Drug-Loaded Polymeric Micelles in the Angiogenic Cascade***

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**Inhibitory effect of paclitaxel and rapamycin individual and dual drug polymeric micelles in the angiogenic cascade.**

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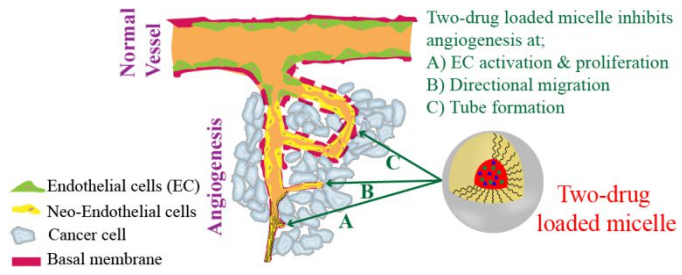
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## Abstract

Angiogenesis is an essential process for disease progression in many solid tumors. There are several major cascade events in the angiogenic process that can be targeted to inhibit new blood vessel formation in the tumor tissue. The purpose of this work is to evaluate the inhibitory effect of paclitaxel (PTX) and rapamycin (RAP) as individual and in dual drug loaded poly(ethylene glycol)-*block*-poly(*d,l* lactic acid) (PEG-*b*-PLA) micelles on the angiogenic cascade processes of proliferation, migration and tube formation. PEG-*b*-PLA PTX and/or RAP micelles were formed and characterized for size and drug loading. Sizes of individual and dual drug micelles were below 40 nm. PEG-*b*-PLA micelles significantly enhanced the aqueous solubility of PTX 1.80 mg/ml and RAP 1.60 mg/ml. The PTX-RAP dual drug PEG-*b*-PLA micelles were able to load PTX and RAP at 1.60 mg/ml for both drugs. Cell proliferation, apoptosis, tubule formation and migration studies were performed in Human Umbilical Vein Endothelial Cells (HUVEC). PTX and RAP in DMSO inhibited HUVEC proliferation with IC<sub>50</sub> values of  $0.82 \pm 0.02$  nM and  $13,829 \pm 681$  nM respectively, while the combination of both drugs in DMSO produced synergistic inhibition. PTX and RAP individual micelles had IC<sub>50</sub> values of  $6.3 \pm 1.1$  nM and  $14,051 \pm 821$  nM respectively. PTX and dual drug micelles had a synergistic inhibition effect on HUVEC proliferation through the induction of apoptosis via Caspase 3/7 activity. *In vitro* tube formation assay demonstrated significant inhibition of tube formation upon treatment with dual drug micelles as compared to individual PTX or RAP micelles. Migration studies in HUVEC have shown that individual PTX micelles inhibited cell migration at 1 nM, while RAP micelles did not show any inhibitory effect on cell migration. Interestingly, the presence of RAP in the dual drug micelles was able to initiate the inhibition of the migration of HUVEC at 0.1 nM concentration of PTX. These results indicate that PTX-RAP dual drug micelles have antiangiogenic effects *in vitro* mediated through three major events in the angiogenic process, and have strong potential for further development as antiangiogenic chemotherapy.

Keywords: Antiangiogenesis, polymeric micelles, combination therapy

## Introduction

Angiogenesis is a process which primarily involves the generation of new blood vessels from preexisting vasculature<sup>1</sup>. The angiogenic process consists of a cascade of events starting with the release of proangiogenic factors by the diseased or the injured cells and ending with the formation of new functional blood vessels. Angiogenesis is primarily regulated through the balance between pro and antiangiogenic molecules<sup>2</sup>. Dysregulated growth of tumor cells results in an angiogenic switch, which causes angiogenesis to compensate for the enhanced requirement of nutrients and oxygen<sup>3</sup>. Proangiogenic factors such as VEGF, bFGF, TGF $\alpha$  &  $\beta$  are mainly secreted from the tumor cells and play an essential role in the tumor growth. Antiangiogenic therapies inhibit the growth of tumor vasculature and control dysregulated tumor growth and metastasis<sup>4</sup>. Normalization of tumor angiogenesis process by antiangiogenic agents has demonstrated effectiveness in cancer therapy<sup>5</sup>. Three major events in the activated angiogenic cascade are endothelial cell proliferation, endothelial cell migration and endothelial cell tube formation<sup>6</sup>. Antiangiogenic inhibitors disrupt one or more of these events partially or fully. Many therapeutic agents such as taxanes and mammalian target of rapamycin (mTOR) inhibitors have both cytotoxic and secondary antiangiogenic effects in tumor tissues. These dual capacities are not fully manifested, due in large part to limitations in dosing regimens and available drug formulations<sup>7-10</sup>.

Rapamycin (RAP) and Paclitaxel (PTX) have demonstrated effectiveness as antiangiogenic agents<sup>9, 11</sup>. PTX is a cytotoxic agent which causes stabilization of microtubule dynamics leading to cell death<sup>12</sup>. It inhibits the endothelial cell proliferation, tube formation and migration<sup>11, 12</sup>. RAP is poorly water soluble macrolide antibiotic which was initially obtained from *Streptomyces hygroscopicus*<sup>13</sup>. It has demonstrated effectiveness in inhibiting the angiogenic process by acting on endothelial cell function<sup>14</sup>. The probable mechanisms through which it exerts effect are directly acting on mTOR, inhibiting VEGF expression and blocking the endothelial to mesenchymal transition<sup>14</sup>. RAP inhibits the mTOR activity by forming complex with FKBP-12 and causes cell cycle arrest at G1 phase<sup>15</sup>. The inhibition of mTOR activity results in the suppression of proangiogenic factors through down regulation of hypoxia mediated pathways<sup>9, 16, 17</sup>.

Polymeric micelles composed of amphiphilic block copolymers form a core-shell structure and provide an excellent platform for the delivery of hydrophobic therapeutic agents in the core of

the micelle<sup>18, 19</sup>. The inner core is protected by the hydrophilic shell that minimizes the non-specific uptake through RES and thereby enhances the circulation time<sup>20</sup>. Polyester based micellar formulations like PEG-*b*-PLA are currently in phase II clinical trials for the treatment of cancer. Currently marketed intravenous formulations of PTX (Taxol<sup>®</sup>) utilize Cremophor EL<sup>®</sup> (CrEI) and ethanol for solubilization<sup>21</sup>. Earlier studies have shown that CrEI formulations cause hypersensitivity reaction in 40% of patients, which limits its use<sup>22</sup>. Additional formulation challenges such as stability and drug-drug compatibility need to be considered in multiple drug delivery in a single dosage form. Given the limitations of surfactants in terms of side effects an alternate delivery system is needed. PEG-*b*-PLA micelles provide an attractive, biocompatible platform for co-solubilization and delivery of multiple hydrophobic molecules with minimal vehicle associated side effects<sup>23, 24</sup>.

In recent studies it was found that multiple drug loaded micelles provide synergistic antitumor efficacy without significantly enhancing the acute toxicity<sup>23-25</sup>. It was also reported that combination chemotherapy can act on multiple pathways to provide enhanced efficacy and reduced toxicity<sup>25, 26</sup>. In earlier investigations of PTX and RAP in tumor cell lines it was shown that RAP can potentiate the effect of PTX, tamoxifen and cisplatin by acting on the mTOR pathway<sup>27-29</sup>. Given that PTX and RAP act by different mechanisms to treat cancer cells, combination therapy with these drugs offers a viable therapeutic option. In addition, both PTX and RAP have demonstrated antiangiogenic effects<sup>9, 11</sup>. However, the combined antiangiogenic response of PTX and RAP has never been studied in endothelial cells. A PEG-*b*-PLA based polymeric micellar system of PTX and RAP offers the ability to target both tumor tissue and endothelial cells by different mechanisms of action in one formulation. Thus, our main objective is to evaluate the antiangiogenic activity of PTX and RAP individual and dual drug micelles in HUVEC cells on the four cellular processes that are essential for angiogenesis which include proliferation, apoptosis, tube formation and migration process<sup>30, 31</sup>.

### **Materials and Methods:**

PTX and RAP were purchased from LC Laboratories (Woburn, MA). HUVEC cells and endothelial growth medium 2 were purchased from PromoCell (Heidelberg, Germany). Cells were cultured as per the manufacturer instructions and experiments were performed between passages 2 and 6. Diblock copolymers PEG<sub>2000</sub>-*b*-PLA<sub>1800</sub> (Mn = 3800, Mw = 4100 and PI =

1.1) and PEG<sub>4000</sub>-*b*-PLA<sub>2200</sub> (Mn = 6100, Mw = 6500 and PI = 1.06) were purchased from Advanced Polymer Materials Inc. (Montreal, CAN). CellTiter-Blue<sup>®</sup> Cell Viability Assay kit and Apo-ONE<sup>®</sup> Homogeneous Caspase-3/7 Assay kit were obtained from Promega Inc. (Madison, WI). All other reagents of analytical grade were purchased from VWR International, LLC. (Radnor, PA) and Fisher Scientific Inc. (Fairlawn, NJ).

## **Methods:**

**Preparation of drug loaded micelles:** PTX, RAP and PTX-RAP dual drug loaded PEG-*b*-PLA micelles were prepared by solvent casting method as reported previously<sup>23, 24, 32</sup>. Briefly, for the preparation of PTX or RAP individual micelles, 15 mg polymer (PEG<sub>2000</sub>-*b*-PLA<sub>1800</sub>) and 2 mg of PTX or RAP was dissolved in 0.5 ml of acetonitrile, which was evaporated under reduced pressure to form a thin polymeric film. Micelles were obtained by rehydration of polymeric film with 0.5 ml deionized water. For the dual drug micelle, PTX (2 mg) and RAP (2 mg), and PEG<sub>2000</sub>-*b*-PLA<sub>1800</sub> (15 mg) polymer were dissolved in 0.5 ml acetonitrile and the micelles were prepared as mentioned above. A second set of PTX or RAP or dual drug micelles of were prepared using PEG<sub>4000</sub>-*b*-PLA<sub>2200</sub> polymer using the same procedure.

**Particle size analysis:** Particle size of polymeric micelles was measured by dynamic light scattering using a Malvern Nano ZS (Malvern Instruments Inc., U.K.). Samples were diluted 20 times with deionized water to yield final polymer concentration of 1.5 mg/ml. The intensity of He-Ne laser (633 nm) was measured at 173°. All measurements were performed at 25 °C after pre-equilibration for 2 min. The particle size was measured in triplicates and Z-average size was reported as the mean with standard deviation and polydispersity index.

**Reverse phase high performance liquid chromatography (RP-HPLC) analysis:** The drug loading was determined using Shimadzu HPLC system consisting of LC-20 AT pump and SPD M20 a diode array detector. The analysis was performed using Zorbax C8 Column (4.6×75mm, 3.5 µm) in isocratic mode with acetonitrile/water (62/38) containing 0.1% phosphoric acid and 1% methanol at a flow rate of 1 ml/min and injection volume of 10 µL. Column temperature was kept at 40 °C. The PTX and RAP peaks were monitored at 227 and 279 nm respectively. The

retention times for PTX and RAP were 1.7 and 5.7 min respectively. All measurements were performed in triplicates.

**HUVEC cell proliferation assay:** HUVEC cells were seeded at the density of 5,000 cells/well in 96 well plates and allowed to attach for 48 h at 37 °C. After incubation, cells were treated with different concentrations of PTX and/or RAP in DMSO (final concentration of DMSO was 0.1%) or individual or dual drug loaded micelles. PTX concentration range was 0.5 pM-50 µM while RAP concentration range was 5.0 nM- 50 µM in the DMSO treatments. The same concentrations were used for PTX/RAP combination in DMSO. PTX concentration range was 10 pM-10 µM while RAP concentration range was 10 nM- 50µM in individual micelle treatments. While for the dual drug loaded micelles PTX:RAP (1:1) molar ratio the concentration range for each drug was from 0.1-50 nM. Cell viability was determined after 48 h by treatment with 20 µL of CellTiter-Blue<sup>®</sup> reagent followed by one hour of incubation at 37 °C and fluorescence (560<sub>Ex</sub>/590<sub>Em</sub>) signal was measured. The drug concentration at 50% growth inhibition (IC<sub>50</sub>) was determined by log(inhibitor) vs. response -Variable slope using GraphPad Prism version 5.04 for Windows, GraphPad Software, San Diego California USA, [www.graphpad.com](http://www.graphpad.com):

$$Y = Bottom + \frac{Top - Bottom}{1 + 10^{((LogIC_{50} - X)(Hillslope))}}$$

Where;  $Y$  is response,  $X$  is concentration,  $Bottom$  is the maximum inhibition,  $Top$  is maximum response,  $Hillslope$  is the Hill slope of the dose response curve.

**Combination Index (CI) analysis:** The combination effect of PTX and RAP on HUVEC cells proliferation was evaluated for drugs solubilized in DMSO or dual drug loaded micelles (see *HUVEC cell proliferation assay* section). Compusyn software<sup>33</sup> was used for the data analysis. This software is based on Chou and Talay median-effect method<sup>34</sup>, in which the median-effect equation is a general equation for dose-effect relationship derived from the mass-action law principle that takes into account the potency and the shape of dose-effect curve. The dose-effect relationship as shown by the mass action law is mathematically described below:

$$\frac{f_a}{f_u} = \left( \frac{D}{D_m} \right)^m$$



Where  $f_a$  and  $f_u$  represent the effect while  $D$  is the dose causing the effect. The dose effect curve can be linearized by the median effect plot where  $x = \log(D)$  and  $y = \log(f_a/f_u)$

$$\log\left(\frac{f_a}{1-f_a}\right) = m\log(D) - m\log(D_m)$$

$f_a$ : the fraction of cells affected upon drug treatment

$f_u$ : the fraction of cells unaffected upon drug treatment,  $(1-f_a)=f_u$

$D$ : the dose of the drug

$D_m$ : the dose that is required to produce a median effect (e.g.,  $IC_{50}$ ,  $ED_{50}$ , or  $LD_{50}$ )

$m$ : the slope of the line

CI value obtained from the software represents the effect of combination. CI value of 1 indicates additive effect,  $CI > 1$  indicates antagonism and  $CI < 1$  indicates synergism. CI value of PTX and RAP were computed using the following formula:

$$CI = \frac{(D)_1}{(Dx)_1} + \frac{(D)_2}{(Dx)_2}$$

Where  $(D_{x1})$  and  $(D_{x2})$  are the inhibitory concentration of drug 1 and drug 2 alone respectively.  $(D)_1$  and  $(D)_2$  are the drug 1 and 2 concentration respectively. The data was represented as  $F_a$ -CI Plot (Chou-Talalay Plot) a plot of CI on y-axis as a function of effect level ( $F_a$ ) on the x-axis.

**Apoptosis assay, Caspase 3/7 activity:** HUVEC cells were plated in 96 well plates at a seeding density of 5,000 cells/well. After 48 h cells were treated with the same concentrations of PTX or RAP and dual drug loaded micelles that were used in the *HUVEC cell proliferation assay*. Caspase 3 and 7 activity was determined after 48 h by treatment with 100  $\mu$ L of Caspase reagent<sup>®</sup>. After two hour of incubation at room temperature luminescence activity was measured. The compiled data was presented as mean and standard deviation of the mean (mean  $\pm$  SD). Significant differences between treatment group means were evaluated using one way ANOVA with Dennett method to compare treatment means against control, using a threshold value ( $\alpha$ ) of 0.05.

**In vitro endothelial tube formation assay:** Matrigel was thawed overnight at 4 °C in the ice bath and then 50  $\mu$ L of solution was used to coat 96 well plates. The plates were then incubated at 37 °C for 60 minutes to ensure complete gelation of the matrix. HUVEC cells were then seeded into 96 well plates at a cell density of 20,000 cells/well and allowed to incubate for 18 h

at 37°C. The total tube length and area were quantified using NIH ImageJ analysis software<sup>35</sup>. Cells were treated with different concentrations of PTX (0.01, 0.1 and 1 nM), RAP (10, 100 and 1000 nM) individual and dual drug micelles of PTX and RAP. The concentrations chosen for this study utilized the individual drugs below their respective IC<sub>50</sub> values as determined by the cell proliferation data.

**Migration Assay:** HUVEC cell migration process was analyzed using xCELLigence RTCA DP instruments (Roche Applied Sciences, Germany). The system measures the electrical impedance which indicates the number of cells that migrated from the apical to the basolateral chamber in response to a chemoattractant. A change in electrical impedance was recorded in terms of *cell index* number. CIM-Plates 16 were coated with 20 µg/ml of fibronectin for 1 h. HUVEC cells were starved for 4 h with serum free medium and seeded on pre-coated fibronectin plates at a density of 15,000 cells/well. A change in electrical impedance was monitored every 10 min for 48 h. In the basolateral chamber, HUVEC cells complete medium was used as control and PTX, RAP and dual drug loaded micelles in complete medium as treatment groups were added in quadruplicates. The compiled data was presented as mean and standard deviation of the mean (mean ± SD). Significant differences between treatment group means was evaluated using one way ANOVA with Tukey's Q method for multiple comparisons for equal variances, using a threshold value ( $\alpha$ ) of 0.05.

## **Results:**

**Drug loading and particle size analysis:** PEG<sub>4000</sub>-*b*-PLA<sub>2200</sub> micelles loaded with PTX were able to solubilize 1.64 mg/ml, while RAP loaded micelles were able to solubilize 1.60 mg/ml. The PTX-RAP dual drug PEG<sub>4000</sub>-*b*-PLA<sub>2200</sub> micelles were able to load PTX and RAP at 1.6 mg/ml for both drugs. RAP loaded PEG<sub>4000</sub>-*b*-PLA<sub>2200</sub> micelles had higher water solubility compared to RAP intrinsic water solubility of 50 µg/ml. Similarly, PEG<sub>2000</sub>-*b*-PLA<sub>1800</sub> micelles resulted in improved water solubility of PTX 1.80 mg/ml in comparison to its intrinsic water solubility of 0.41 µg/ml. However, PEG<sub>2000</sub>-*b*-PLA<sub>1800</sub> with RAP or dual drug micelles were able to initially load drugs at similar concentrations but were not stable post 10 h at 25 °C due to drug(s) precipitation. PTX in PEG<sub>2000</sub>-*b*-PLA<sub>1800</sub> and RAP PEG<sub>4000</sub>-*b*-PLA<sub>2200</sub> individual micelles demonstrated excellent stability at 25 °C for 24 h at 25 °C with more than 95% drug was retained in solution. PEG<sub>4000</sub>-*b*-PLA<sub>2200</sub> dual drug micelles demonstrated higher stability at

25 °C for 24 h in comparison to PEG<sub>2000</sub>-*b*-PLA<sub>1800</sub> dual drug micelles. Stability studies also indicated that the P0EG<sub>4000</sub>-*b*-PLA<sub>2200</sub> PTX micelles also were not stable due to drug precipitation. Therefore, all subsequent experiments were performed using PEG<sub>4000</sub>-*b*-PLA<sub>2200</sub> for RAP or dual drug micelles and PEG<sub>2000</sub>-*b*-PLA<sub>1800</sub> PTX micelles. Both PEG<sub>4000</sub>-*b*-PLA<sub>2200</sub> and PEG<sub>2000</sub>-*b*-PLA<sub>1800</sub> polymers are biocompatible polymers that are not expected to affect the cells directly based on controls that were run in the experiment (data not included).

PEG<sub>2000</sub>-*b*-PLA<sub>1800</sub> PTX micelle sizes were  $19.3 \pm 0.1$  nm (polydispersity index =  $0.128 \pm 0.004$ ), while PEG<sub>4000</sub>-*b*-PLA<sub>2200</sub> RAP and dual drug micelles had sizes of  $35.0 \pm 0.4$  nm (polydispersity index =  $0.138 \pm 0.005$ ) and  $35.0 \pm 0.3$  nm (polydispersity index =  $0.117 \pm 0.009$ ) respectively. All prepared micelles showed unimodal distribution with a polydispersity index value of less than 0.2.

**HUVEC cell proliferation assay:** The antiproliferative effect of PTX, RAP and dual drug micelles were evaluated in HUVEC cells. The cytotoxicity of individual drug and dual drug combination (1:1) in DMSO and in micelles demonstrated a dose dependent decrease in cell viability. The IC<sub>50</sub> values of PTX and RAP in DMSO were  $0.82 \pm 0.02$  nM and  $13,829 \pm 681$  nM respectively (Figure 1A & 1B). We observed a decrease in cytotoxicity with PTX micelles and comparable toxicity in RAP micelles. PTX loaded in PEG<sub>2000</sub>-*b*-PLA<sub>1800</sub> micelle and RAP loaded PEG<sub>4000</sub>-*b*-PLA<sub>2200</sub> micelle showed IC<sub>50</sub> value of  $6.3 \pm 1.1$  nM and  $14,051 \pm 821$  nM respectively (Figure 1C & 1D). The combination of PTX and RAP as free drugs in DMSO (Figure 1C) or dual drug loaded micelles (Figure 1F) demonstrated strong dose dependent inhibition in comparison to individual drug, see *Combination Index (CI) analysis* section. The concentration vs. cell viability plots (Figures 1C and 1F) for the combination of drugs in DMSO and in micelles plots the total drug concentration of PTX + RAP against the cell viability.

**Combination Index (CI) analysis:** To further analyze whether PTX and RAP combination are synergistic, additive or antagonistic, against HUVEC proliferation, the combination indices for the various dosing ratios were calculated using Compusyn software. The calculated CI of PTX and RAP in DMSO as well as dual drug micelles were well below 1.0 (Figure 2A & 2B) indicating significant synergistic antiproliferative effect against the HUVEC.

**Apoptosis assay, Caspase 3/7 activity:** Our results indicate that PTX loaded PEG<sub>2000</sub>-*b*-PLA<sub>1800</sub> micelles produced concentration dependent apoptosis in HUVEC cells. PTX loaded PEG<sub>2000</sub>-*b*-PLA<sub>1800</sub> micelles at 10 nM concentration induced apoptosis in HUVEC cells. However, lower concentrations of PTX micelles (<10 nM) did not induced apoptotic activity (Figure 3). In addition, RAP loaded PEG<sub>4000</sub>-*b*-PLA<sub>2200</sub> micelles at concentration range 10-20,000 nM did not induce apoptosis in HUVEC cells (data is not shown). Interestingly, we observed that with dual drug micelles PTX:RAP (1:1) molar ratio, RAP induces the apoptotic activity of PTX at 0.01 nM and significantly increases the apoptotic activity of PTX at 10 nM when compared to the individual PTX micelles at 0.01 nM and 10 nM (Figure 3).

***In vitro* endothelial tube formation assay:** HUVEC were treated and cell differentiation was monitored *in vitro* by tube formation on matrigel matrix. HUVEC cells without treatment resulted in formation of regular capillary like tubular structures (Figure 4A). PTX loaded PEG<sub>2000</sub>-*b*-PLA<sub>1800</sub> micelles at 1, and 0.1 nM (Figure 4B) reduced the tube formation by  $58.9 \pm 11.7\%$  and  $36.7 \pm 10.1\%$  respectively while PTX micelles at 0.01 nM showed no significant reduction in the tube formation area compared to control. RAP loaded PEG<sub>4000</sub>-*b*-PLA<sub>2200</sub> micelles at 1000 nM and 100 nM, (Figure 4C) showed reduction in tube formation areas of  $69.7 \pm 10.1\%$  and  $23.2 \pm 19.6\%$  respectively while RAP micelles at 10 nM showed no reduction in the tube formation area compared to control. Overall, PTX loaded polymeric micelles demonstrated greater inhibition of tube formation at lower doses in comparison to RAP polymeric micelles. These result trends were similar to those seen in the cell proliferation data. The dual drug micelles demonstrated significant reduction in tube formation process in comparison to PTX or RAP individual micelles. PTX and RAP dual drug PEG<sub>4000</sub>-*b*-PLA<sub>2200</sub> micelles at 0.05 nM and 50 nM respectively showed reduction in the tube formation area by  $78.8 \pm 7.6\%$  (Figure 4D).

**Migration assay:** To assess the effect of drug loaded polymeric micelles on endothelial cell migration, real time migration using xCELLigence RTCA DP Instrument was used. The cell index value indicates the number of cells that migrate in response to a chemoattractant. By plotting the *cell index* values over *time*, a signature real-time cellular migration (RTCM) profile can be generated to monitor HUVEC migration in real time. We observed dose-dependent significant inhibition of HUVEC migration with PTX loaded PEG<sub>2000</sub>-*b*-PLA<sub>1800</sub> micelles at 1

nM, while no significant inhibitory effect on the migration process was seen for PTX micelles at 0.1 nM and 0.01 nM PTX concentrations (Figure 5A). In contrast, RAP PEG<sub>4000</sub>-*b*-PLA<sub>2200</sub> micelles did not show any significant inhibitory effect even at 20,000 nM (Figure 5B). Interestingly, in case of dual drug polymeric micelles, PTX at fixed concentration of 0.1 nM with different concentration of RAP (5,000, 10,000 and 20,000 nM) showed significant inhibition in cell migration process at all RAP concentrations as shown in (Figure 5C). These findings confirm the synergistic/additive effect of dual drug polymeric micelles on HUVEC cell migration process.

### **Discussion:**

Antiangiogenic therapies for the treatment of tumor have gained considerable interest in recent times<sup>36</sup>. It was presumed that genetically stable endothelial cells are less likely to result in failure of therapy due to acquired resistance. However, clinical studies have shown that antiangiogenic therapies can acquire resistance over short period of time<sup>37</sup>. The suggested mechanisms for resistance include up regulation of compensatory antiangiogenic mediators, and enhanced ability of tumor cells to survive hypoxia. Therefore, there is a critical need to develop a new treatment modality that can prevent the antiangiogenic treatment resistance. In this study, for the first time we have demonstrated that combined delivery of PTX and RAP can provide synergistic antiangiogenic activity in endothelial cells. PTX and RAP act on different pathways to provide antiangiogenic effect<sup>11, 12</sup>. It has already been demonstrated that combination therapy of chemotherapeutics can provide a synergistic response and also reduce the toxicity associated with higher doses of individual drugs<sup>23</sup>. Since there is clinical evidence for combination therapy in tumor treatment, it can be postulated that a similar approach in developing antiangiogenic agents will help overcome resistance to treatment in endothelial cells. For both, chemotherapeutics and antiangiogenesis multiple drug combinations in a single delivery system is not yet commercially available. Based on these findings we have selected polymeric micelles as a vehicle for combined delivery of PTX and RAP. It was observed that PEG-*b*-PLA micelles considerably improved the water solubility of PTX and RAP<sup>24</sup>. Two different polymers of different molecular weight and block length i.e. PEG<sub>4000</sub>-*b*-PLA<sub>2200</sub> and PEG<sub>2000</sub>-*b*-PLA<sub>1800</sub> were selected for the study. It was earlier reported that PTX loaded PEG<sub>4000</sub>-*b*-PLA<sub>2200</sub> are not physically stable<sup>24</sup>. Therefore, we have selected PEG<sub>2000</sub>-*b*-PLA<sub>1800</sub> for the preparation of PTX

loaded polymeric micelles due to enhanced stability in comparison to PEG<sub>4000</sub>-*b*-PLA<sub>2200</sub> micelles<sup>32</sup>. PTX loaded PEG<sub>2000</sub>-*b*-PLA<sub>1800</sub> micelles displayed less precipitation presumably due to better drug-polymer compatibility. Similarly, PEG<sub>4000</sub>-*b*-PLA<sub>2200</sub> was used for RAP and dual drug loaded micelles as enhanced stability was observed at room temperature due to better drug(s)-polymer interaction. We observed similar drug loading for dual drug loaded micelles as compared to the individual drug loaded micelles. The ability of the block copolymers to load two drugs into the core at same concentrations as individual drugs is a behavior that needs further study, but these findings are consistent with earlier investigators<sup>23, 24</sup>.

RAP and dual drug loaded micelles were larger in size  $\approx$  35 nM in comparison to PTX loaded micelles  $\approx$  20 nM. This difference in size was observed due to differences in the copolymer block lengths and molecular weights. Other investigators using different block copolymer for the preparation of micelles have reported similar behavior<sup>38</sup>. It was reported that block copolymers of high molecular weight result in micelles of higher hydrodynamic radii. According to our findings, PEG<sub>4000</sub>-*b*-PLA<sub>2200</sub> always formed micelles of larger diameter irrespective of individual or multiple drug loaded micelles as compared to PEG<sub>2000</sub>-*b*-PLA<sub>1800</sub>. Therefore, the block copolymer chosen plays a significant role in determining the size of polymeric micelles formed.

*In vitro* cell proliferation results for drugs in DMSO and micelles indicate that PTX is more potent in inhibiting the HUVEC cell proliferation in comparison to RAP and these results are in agreement with earlier published reports<sup>39, 40</sup>. Dual drug micelles of PTX:RAP in (1:1) molar ratio displayed synergistic drug response as observed through combination index analysis (Figure 2B). Similar findings were reported for PTX and RAP drug combinations in different tumor cell lines<sup>23</sup>. It has been suggested that RAP potentiates the cytotoxicity of PTX by inhibiting mTOR pathway<sup>41</sup>. The drug loaded micelles displayed higher IC<sub>50</sub> values in comparison to drug alone (Figure 1). This difference in IC<sub>50</sub> values is possibly due to the high stability of the micelles *in vitro* which results in lower free drug being available to exert its effect on HUVEC cells. PTX loaded PEG<sub>2000</sub>-*b*-PLA<sub>1800</sub>, RAP loaded PEG<sub>4000</sub>-*b*-PLA<sub>2200</sub> and dual drug PEG<sub>4000</sub>-*b*-PLA<sub>2200</sub> micelles displayed dose dependent response on HUVEC cells. The combination of the PTX and RAP as free drugs or in dual drug micelle synergistically inhibited HUVEC cell proliferation over wide range of dosing (Figure 2). PTX and RAP dual drug

micelles demonstrated strong apoptotic activity in comparison to PTX loaded micelles (Figure 3). RAP loaded PEG<sub>4000</sub>-*b*-PLA<sub>2200</sub> micelles did not exhibit apoptotic activity. The mechanism by which RAP enhances the apoptotic activity of PTX is poorly understood. However, it has been postulated that RAP inhibits S6K1 phosphorylation which in turn inactivates pro-apoptotic molecules<sup>41</sup>. Our observation is consistent with earlier findings by Shafer et al. which suggest that RAP, a cytostatic agent, potentiates the apoptotic effect of PTX in endometrial cancer cells<sup>41</sup>. Thus our data suggests that PTX and RAP polymeric micelle based combination therapy can enhance the antiangiogenic effect of these cytotoxic chemotherapeutic agents.

*In vitro* tube formation experiment PTX and RAP concentrations selected for the study were below the IC<sub>50</sub> values of the drugs in polymeric micelles. This selection was based on earlier findings that these chemotherapeutic agents can exert antiangiogenic effect at much lower dose than their IC<sub>50</sub><sup>42</sup>. The data indicates that dual drug loaded micelles significantly reduce the tube formation in comparison to individual drug loaded micelles (Figure 4). Endothelial tube formation involves multiple steps such as attachment, and migration prior to tube formation process. Tube formation is initiated with attachment of endothelial cells on the basement matrix and then is followed by migration of these cells towards each other to eventually form tubes<sup>6</sup>. Our data has shown this process can be inhibited at concentrations well below the IC<sub>50</sub> value of PTX and RAP and dual drug micelles (Figure 4). Other studies done with Taxol<sup>®</sup> (commercial formulation of PTX) or RAP inhibit capillary formation process *in vitro* at much lower concentrations than the IC<sub>50</sub> value of individual drugs, which is consistent with our findings<sup>43</sup>. Similar to the migration results, PTX was more potent than RAP in inhibiting the tube formation process. It was reported that PTX can cause cell cycle arrest in S phase or G1/S phase at lower concentration which is primarily responsible for inhibiting the tube formation process<sup>42</sup>. In dual drug micelles significant inhibition of HUVEC tube formation was observed presumably due to PTX and RAP acting on different pathways in the endothelial cells to produce a synergistic effect. This hypothesis can be substantiated by earlier findings that demonstrate that RAP can potentiate the cytotoxic effect of PTX in the cell proliferation study<sup>27</sup>. The synergistic effect of PTX and RAP dual drug micelles suggests that targeting multiple pathways through the combination strategy will enhance the antiangiogenic effect.

Real time migration assay with the dual drug micelles demonstrated synergistic/additive inhibition in HUVEC cell migration (Figure 5). It was observed that PTX loaded PEG<sub>2000</sub>-*b*-

PLA<sub>1800</sub> micelles at 0.01 nM concentrations and RAP loaded PEG<sub>4000</sub>-*b*-PLA<sub>2200</sub> micelles at different concentration (5,000, 10,000 and 20,000 nM) did not showed inhibition in the endothelial cell migration process (Figure 5A & 5B). However, dual drug micelles of PTX 0.01 nM and RAP at 5000 nM, 10,000 nM or 20,000 nM demonstrated significant inhibition in cell migration (Figure 5C). These results suggest that the combined treatment of PTX and RAP inhibits HUVEC cell migration by synergistic/additive response. Cell migration process is regulated through reorientation of centrosome in the intended direction of movement<sup>44</sup>. It was also observed that change in microtubule plasticity can alter the reorientation of the centrosome<sup>44</sup>. Based on this mechanism, in our study we postulate that RAP potentiates the antimigratory effect of PTX on endothelial cells (Figure 5) by changing the microtubule plasticity. We observed that RAP enhanced the antimigratory activity of PTX at 0.01 nM a concentration at which individual PTX micelles did not inhibit endothelial cell migration. Further studies are required to delineate the exact molecular mechanism behind the enhanced migratory activity in the case of dual drug micelles. Our study provides strong evidence that combined treatment of PTX and RAP dual drug loaded micelles is advantageous in comparison to individual drugs for antiangiogenic treatment due to its inhibition of three major cascade events in the angiogenic process.

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## Legend for Figures

Figure 1: HUVEC viability compared to control (untreated cells). Cells were treated with: PTX in DMSO (A), RAP in DMSO (B), PTX micelles (C) and RAP micelles (D). (Mean  $\pm$  SD, n = 4).

Figure 2: *Fa-CI* plots of PTX and RAP combination in HUVEC cells. PTX and RAP in DMSO (A), dual drug loaded PEG<sub>4000</sub>-*b*-PLA<sub>2200</sub> micelles (B). (Mean  $\pm$  SD, n = 4).

Figure 3: Caspase 3/7 activity of HUVEC cells untreated, treated with PTX micelles (0.01 and 10 nM) and dual drug micelles PTX:RAP (1:1) at 0.01 and 10 nM corresponding to PTX concentration. \* Represents significant difference from untreated control. (Mean  $\pm$  SD, n=3).

Figure 4: HUVEC tuber formation: Untreated-control (A), PTX micelle 0.1 nM (B), RAP micelle 100 nM (C), Dual drug micelle PTX 0.05 nM & RAP 50 nM (D).

Figure 5: Real-time cellular migration profile (RTCM) for HUVEC cells treated with: PTX micelles (A), RAP micelles (B), and dual drug loaded micelle with PTX 0.1 nM and different RAP concentrations (C). \* Represent significant difference from untreated control. (Mean  $\pm$  SD, n=4)

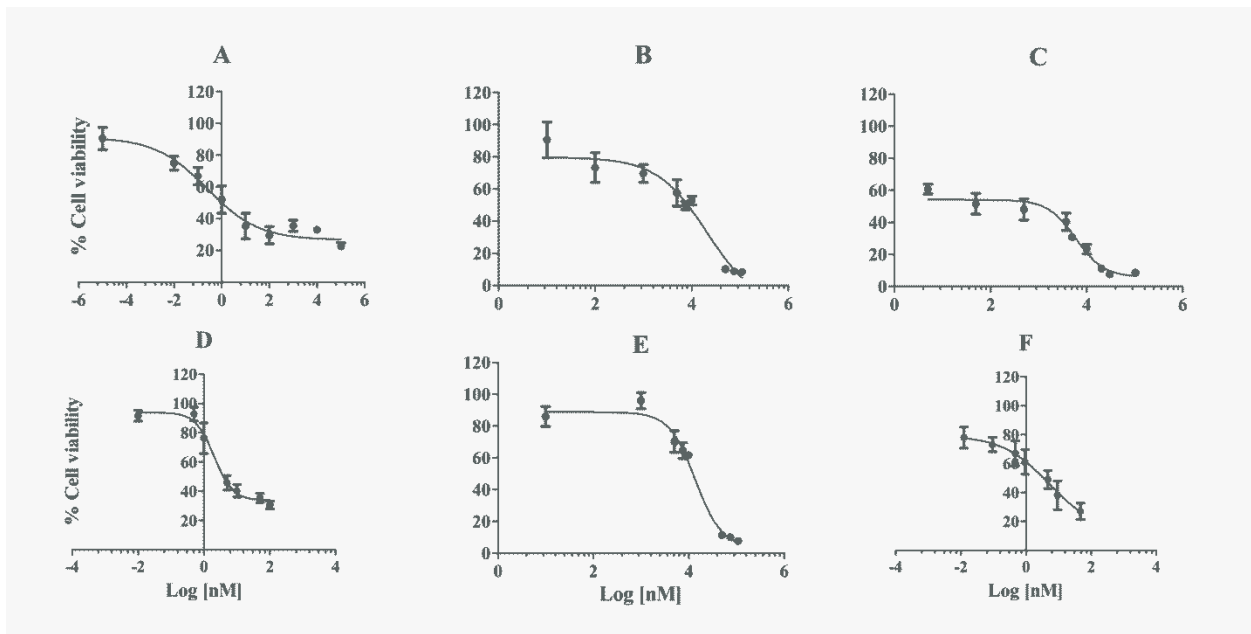


Figure 1: HUVEC viability compared to control (untreated cells). Cells were treated with: PTX in DMSO (A), RAP in DMSO (B), PTX/RAP combination in DMSO (C), PTX micelles (D), RAP micelles (E) and PTX/RAP dual drug loaded micelles (F). (Mean  $\pm$  SD, n = 4).

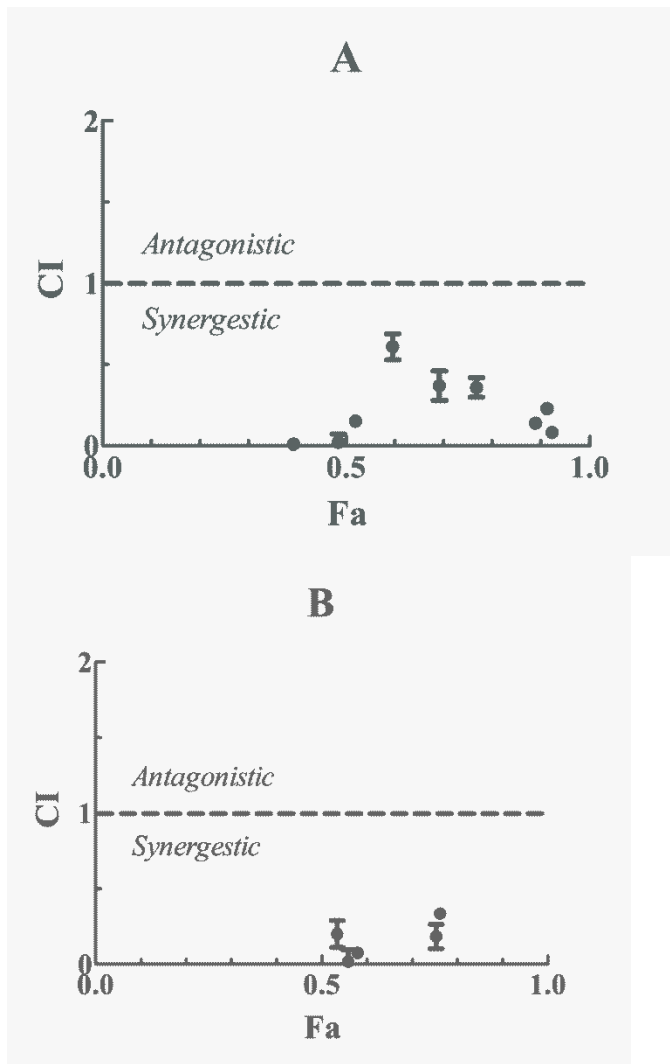


Figure 2: *Fa-CI* plots of PTX and RAP combination in HUVEC cells. PTX and RAP in DMSO (A), dual drug loaded PEG<sub>4000</sub>-*b*-PLA<sub>2200</sub> micelles (B). (Mean ± SD, n = 4).

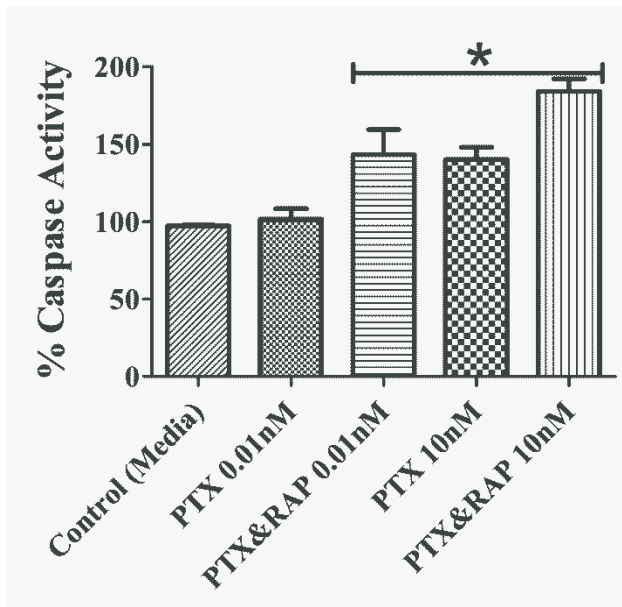


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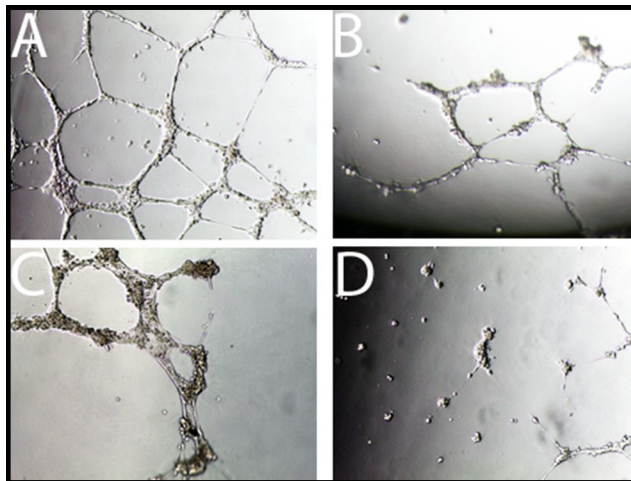


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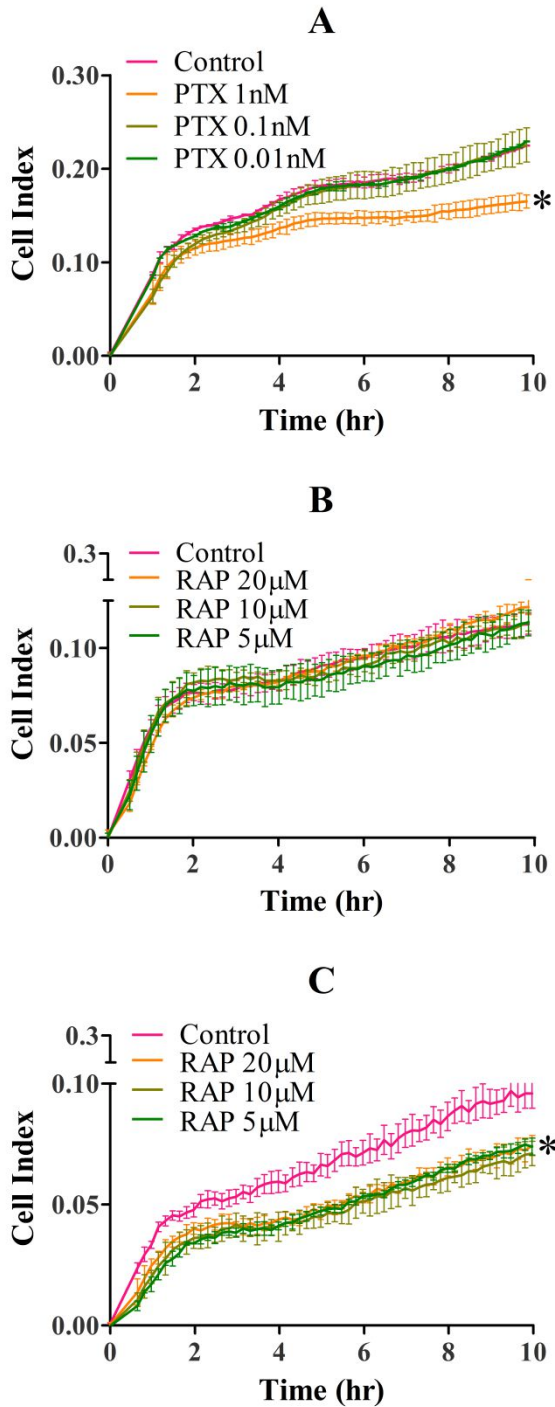


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