

Increased Tumor Uptake of Chemotherapeutics and Improved Chemoresponse by Novel Non-anticoagulant Low Molecular Weight Heparin

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Abstract. *Background:* Recent prospective clinical trials of low molecular weight heparins (LMWHs) have demonstrated that these agents may provide significant advantages in terms of progression-free and overall survival in certain subgroups of cancer patients. The mechanisms of improved survival associated with LMWHs are not known, and may involve direct and/or indirect effects on tumor growth. The purpose of this study was to investigate the effects of LMWH and a sulfated non-anticoagulant LMWH (S-NACH) on tumor chemotherapeutic uptake and chemoresponse. *Materials and Methods:* LMWH and S-NACH were tested for their ability to reduce tumor growth and tumor-associated angiogenesis using three different *in vivo* models. Biodistribution studies were undertaken to determine the effect of these agents on uptake of paclitaxel (PACL) and doxorubicin (Dox) by breast cancer tumor xenografts. *Results:* LMWH and S-NACH (10 mg/kg *s.c.* daily) effectively limited tumor growth of human A549 lung adenocarcinoma xenografts in the nude mouse. In an MDA453/LCC6 breast tumor xenograft model, PACL plus S-NACH showed significant ($p < 0.01$) tumor growth suppression and improved survival when compared to PACL alone. LMWH increased [¹²⁴I]-PACL uptake into

MDA453/LCC6 tumors, with tumor:muscle ratios several fold greater than that of [¹²⁴I]-PACL alone 24 h post-injection. Similarly, LMWH and S-NACH significantly ($p < 0.01$) increased the uptake of Dox by 1.5-2 fold in MCF7 Dox-resistant tumor xenografts. *Conclusion:* Protocols utilizing adjuvant or neo-adjuvant therapy with LMWH or S-NACH could lead to increased tumor chemo responsiveness, potentially overcoming tumor chemoresistance.

A broad spectrum of clinically significant hemostatic abnormalities may afflict as many as 15-25% of cancer patients. Furthermore, hemostatic complications are the second most common cause of mortality in cancer patients, particularly in those with pancreatic, gastrointestinal or lung cancer, and 10% of newly diagnosed myeloma patients treated with any type of chemotherapy develop deep venous thrombosis (1-3). The impact of cancer cells and chemotherapy on the activation of the coagulation cascade is responsible for a pro-thrombotic state found in many cancer patients (4). Various mechanisms related to the activation of the coagulation or fibrinolytic systems in cancer may be involved in tumor development, progression and metastasis. Activation of coagulation can have both systemic and local consequences. The systemic consequences involve deep vein thrombosis or metastasis. Local consequences involve the deposition of fibrin and plasma proteins in the tumor interstitium, resulting at least in part, from tumor vasculature that is inherently leaky. This fibrin deposition results in imposition of the initial tumor structure, regulation of inflammatory cell infiltration, induction of angiogenesis and formation of a mature stroma (5). In addition, accumulation of fibrin and other plasma proteins in the tumor

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microenvironment contributes significantly to increased interstitial pressure that impedes the penetration of chemotherapeutic agents into the tumor (6-8). Tumor-generated polymerized fibrin also results in the formation of a physical barrier protecting the tumor from natural killer cells and other exogenous anticancer agents.

Tissue factor (TF) has been characterized best for its role in blood coagulation, but recent studies have suggested a role for TF in physiologic processes distinct from hemostasis (9-11). TF is frequently overexpressed upon progression from a benign to malignant phenotype, and is associated with aggressive behavior and poor outcomes in some types of tumors (12). TF is also abundantly expressed in newly forming vessels associated with physiological and pathological angiogenesis (13, 14), and has been shown to induce cellular signaling and to promote angiogenesis and tumor metastasis (reviewed in (15)). Initial studies have demonstrated potent anti-angiogenesis and anti-metastasis efficacy for various mechanisms that interfere with TF/VIIa (16-18). Administration of low molecular weight heparin (LMWH) has been shown to induce the localized release of tissue factor pathway inhibitor (TFPI), a key endogenous inhibitor of the TF/VIIa complex, from the endothelium and significantly inhibit angiogenesis (16, 19). Thus, localized expression of a potent TF inhibitor has the potential to play a major role in the control of tumor-induced angiogenesis, and treatments that target this pathological process may result in inhibition of tumor growth and metastasis.

Retrospective analyses of clinical trials in which LMWH had been used to treat cancer patients with established thrombosis have suggested a survival advantage for the treated groups (reviewed in (20)). The first prospective, randomized, double-blind study designed to determine the potential value of long-term LMWH therapy to improve survival in cancer patients suggested a striking survival advantage for LMWH heparin treatment in a subgroup of patients with good-prognosis (21). A second clinical trial in patients with small-cell lung carcinoma showed advantages in terms of progression-free and overall survival for patients who received LMWH for 18 weeks (22). Additional recent studies demonstrated survival advantage in patients without evidence of metastatic disease (23), and in a subgroup of patients with a variety of tumor types (24). In the latter study, the benefits of LMWH therapy were seen for months and years after the period of active administration. These results should stimulate additional clinical trials to develop optimized regimes for treatments with these agents.

The effects of LMWHs on survival of cancer patients may be due to direct or indirect effects on tumor growth and/or angiogenesis. The current study evaluated anti-TF-associated strategies for their ability to reduce tumor growth and tumor-

associated angiogenesis using three different *in vivo* model systems: A549 human lung carcinoma cell-derived tumor implants in the chick chorioallantoic membrane (CAM); A549 tumor xenografts in the nude mouse; and an MDA435/LCC6 breast cancer xenograft model in mice. This study also investigated the effects of LMWHs on the uptake of chemotherapeutic agents into breast tumors. Specifically, two agents were tested: (i) the commercially available LMWH tinzaparin (TINZ), and (ii) an oxidized sulfated ultra-LMWH with limited to no systemic anticoagulant effects but intact intravascular anticoagulant and local antithrombotic effects through the induction of endothelial TFPI release. This sulfated non-anti-coagulant LMWH (S-NACH) has limited effects on hemostasis (25).

Materials and Methods

Cell lines and cell culture. The human non-small cell lung carcinoma cell line A549 (American Type Culture Collection, Manassas, VA, USA) was grown in F-12K medium (Kaighn's Modification of Ham's F-12 medium) containing 2 mM L-glutamine and 1,500 mg/l sodium bicarbonate. MDA435/LCC6 human breast carcinoma cells were cultured as described previously (26). Doxorubicin (Dox)-resistant MCF7 breast cancer cells were cultured in DMEM containing 4 mM L-glutamine, 4,500 mg/l glucose, 1 mM sodium pyruvate, and 1,500 mg/l sodium bicarbonate (Gibco, USA). Media were supplemented with 10% fetal bovine serum (FBS; Sigma, USA), and cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Tumor cells in exponential growth phase were harvested using 0.25% trypsin-EDTA, washed and suspended in medium.

Tumor cell implantation into the CAM. CAM studies were performed as described previously (27) with minor modifications. Ten-day-old chick embryos were obtained from SPAFAS (Preston, CT, USA) and incubated at 37°C with 55% relative humidity. A hypodermic needle was used to make a small hole in the blunt end of the eggshell, with a second hole then made on the broad side of the egg over an avascular portion of the embryonic membrane. Mild suction was applied to the first hole to displace the air sac, so that the CAM dropped away from the shell. Using a Dremel drill (Racine, WI, USA) a 1.0 cm² window was cut in the shell over the false air sac, allowing access to the CAM. To induce tumors, 1×10⁹ A549 human lung adenocarcinoma cells in 20 µl of medium were mixed with 20 µl of Matrigel (BD Bioscience, San Jose, CA, USA) and 10 µl test compound, or PBS (as a control), and then the entire suspension of 50 µl was pipetted onto the branch point of the CAM. On day six after implantation, tumors were harvested and then divided into two sections for tumor weight and hemoglobin (Hb) determinations, respectively.

Determination of tumor Hb levels. Tumor Hb content was indexed as a measure of tumor vascularity. Briefly, tumor sections were placed into a 0.5 ml tube containing double distilled water and then homogenized for 5-10 min. The samples were subjected to centrifugation at 4,000 rpm for 10 min and then the supernatants were collected. A volume of 50 µl of supernatant were mixed with 50 µl of Drabkin's reagent and allowed to sit at room temperature for 15-30 min, after which 100 µl was placed in a 96-well plate and absorbance measured at 540 nm with a Microplate Manager ELISA

reader. Hb concentration was expressed as mg/ml based on comparison with a standard curve.

Lung cancer (A549) xenograft studies. Lung cancer xenograft experiments were carried out in the animal research facility of the Stratton VA Medical Center, Albany, NY, USA. All experiments were performed in compliance with Public Health Service Policy on Humane Care and Use of Laboratory Animals and approved by the VA Medical Center IACUC. Female NCr nude homozygous mice aged 5-6 weeks with body weights of 20 g were obtained from Taconic Farms (Hudson, NY, USA). Animals were maintained under specific pathogen-free conditions and housed four animals per cage, under controlled conditions of temperature (20-24°C) and humidity (60-70%) and a 12 h light/dark cycle. Water and food were provided *ad libitum*. A549 lung adenocarcinoma tumor cells in exponential growth phase were harvested using 0.25% trypsin-EDTA washed and suspended in medium. Cells (10^7 total) in 100 μ l of medium were mixed with the same volume (100 μ l) of Matrigel and then injected subcutaneously (*s.c.*) into the left and right flank regions of each mouse. Mice were weighed daily and tumor volume measurements were performed on implants every other day after inoculation using calipers. Tumor volume was calculated according to the formula ($W \times L^2 / 2$), where W=width and L=length. Mice with tumors of 225-275 mm³ in volume proximal to the injection site were randomized into treatment groups (n=6 per group).

Drug treatments and evaluation of tumor response. Chemotherapeutic drug was administered intraperitoneally (*i.p.*) daily for the course of the experiment or until tumor volume was 2000 mm³ (as per IACUC approval). LMWH compounds were administered *s.c.* daily. At the conclusion of the experiment, all animals were sacrificed in a CO₂ chamber and tumor masses were collected and weighed. Data represent the average weight of all tumor xenografts (right and left sides).

Breast cancer (MDA435/LCC6) xenograft and biodistribution studies. NCr *nu/nu* athymic (Nude) mice 6-10 weeks of age and weighing 20-25 g were obtained from NCI (Frederick, MA, USA) and housed at the Medical Research Complex (MRC) at Roswell Park Cancer Institute, USA. Animal care complied with all Federal and State mandates and the IACUC guidelines. MDA435/LCC6 human breast carcinoma cells were implanted directly from cell cultures harvested by trypsinization and adjusted to 1×10^7 viable cells per ml. A volume of 0.2 ml of each cell suspension was injected *s.c.* into the shoulder region of each mouse (26). Alternatively, tumors were implanted *via* subcutaneous implant as follows: a 50 mg fragment of non-necrotic tumor was implanted *s.c.* using a cancer implant needle (13 gauge \times 3.25"). After four days, mice (in groups of 4-10) were given drugs as described for each experiment.

Drug preparation. Paclitaxel (PACL) was prepared as a 7.5 mg/ml stock solution in equal parts of Cremophor EL (Fluka, Sigma/Aldrich, USA) and absolute ethanol, then further diluted immediately before use in 0.9% NaCl (saline) to the appropriate concentration for intravenous (*i.v.*) administration. PACL (20 mg/kg/inj) was administered once daily every three days starting on day four, for a total of three injections. LMWH compounds were dissolved at 1 mg/ml in saline for *s.c.* injection daily for 14 days (10 mg/kg/inj). For [¹²⁴I]-PACL biodistribution studies, mice were injected with 30-60 μ Ci of labeled drug formulated in saline:Cremophor:ethanol

(5:0.5:0.5) *via* the tail vein. Three mice for each time interval were sacrificed by *i.p.* administration of sodium pentobarbital. Body organs (tumor, muscle, and blood) were removed immediately, weighed, and radioactivity in the organs was measured using a gamma counter. Radioactivity uptake was calculated as the percentage of the injected dose per gram of tissue (%ID/g).

Statistical methods. For each animal, tumor volume (v) was measured using electronic calipers and recorded every 3-4 days. Tumor volume was calculated using the formula: $v = 0.4 (L \times W^2)$. The time in days to the pre-determined target tumor volume of 400 mm³ was linearly interpolated from a plot of log (volume) *versus* time. Statistically significant differences in tumor volumes between control and drug-treated mice were determined by the Cox-Mantel test (26). For the Cox-Mantel test, the time-to-event data for animals that did not reach the target tumor volume, either because of long-term cure (defined as those animals that were still alive at the conclusion of the experiment whose tumors either completely regressed or did not reach the pre-set target volume) or early death due to drug toxicity, were treated as censored data. All statistical tests were two-sided.

Preparation of [¹²⁴I]-PACL. Na¹²⁴I in 0.1 M NaOH (10-30 μ l) was added to 100 μ l of 5% CH₃COOH in CH₃CN, mixed and then transferred to an IODO-GEN coated tube. The stannylated precursor Debenzoyl-3'-(3-trimethylstannylbenzoyl) PACL (40 μ g) was dissolved in a CH₃COOH/CH₃CN (50 μ l of 5%) solution and transferred to the IODO-GEN-coated tube. The solution was mixed and left at room temperature for 15 min. The reaction mixture was purified by HPLC using a Phenomenex Luna C18(2) column (4.6 \times 250 mm). The eluent was 60% CH₃CN, 40% water and the flow rate was 1 ml/min. The product was collected, diluted with water and loaded onto a C18 Sep-Pak cartridge (Waters, Milford, MA, USA). The product was eluted with ethanol. The radiochemical yield of the formulated product was ~40%. The specific activity was found to be >1 Ci/ μ mol for the labeled product. The final product was formulated in saline:Cremophor:ethanol (5:0.5:0.5) for animal studies.

Analysis of Dox uptake by HPLC. Preparation of standard sample: A stock solution of Dox was prepared in acetonitrile (3.0 mg/ml) and stored at -80°C. The stock solution was diluted with 70% acetonitrile to prepare working solutions at a variety of final concentrations. Tissue of the mouse heart, lung and tumor was minced and homogenized with regular saline to generate homogenates in the range of 60-120 mg tissue/ml. Working calibration curves for plasma and tissue were developed by adding stock concentrations of Dox to blank (treatment naive) mouse plasma and homogenates of heart, lung and tumor tissue.

Sample preparation: To determine Dox content, 0.2 ml of plasma or tissue homogenate (tumor, heart, lung) were pipetted into a 2.0-ml polypropylene microcentrifuge tube followed by 0.1 ml of borate buffer (80 mM, pH 9) and 1.5 ml of chloroform/methanol (4:1). The tube was vortexed for 15 min and then centrifuged for 10 min at 20,000 \times g at 4°C. The organic layer was removed and dried by vacuum centrifugation. Dried samples were reconstituted with 150 μ l of 80% acetonitrile and an aliquot of 30 μ l was analyzed by HPLC. Analyses were performed on a reverse-phase HPLC system consisting of a Waters 2695 separation module (Waters Assoc. Milford, MA, USA) coupled to a Waters 2475 multi λ fluorescent detector. The excitation wavelength was set at 480 nm and emission

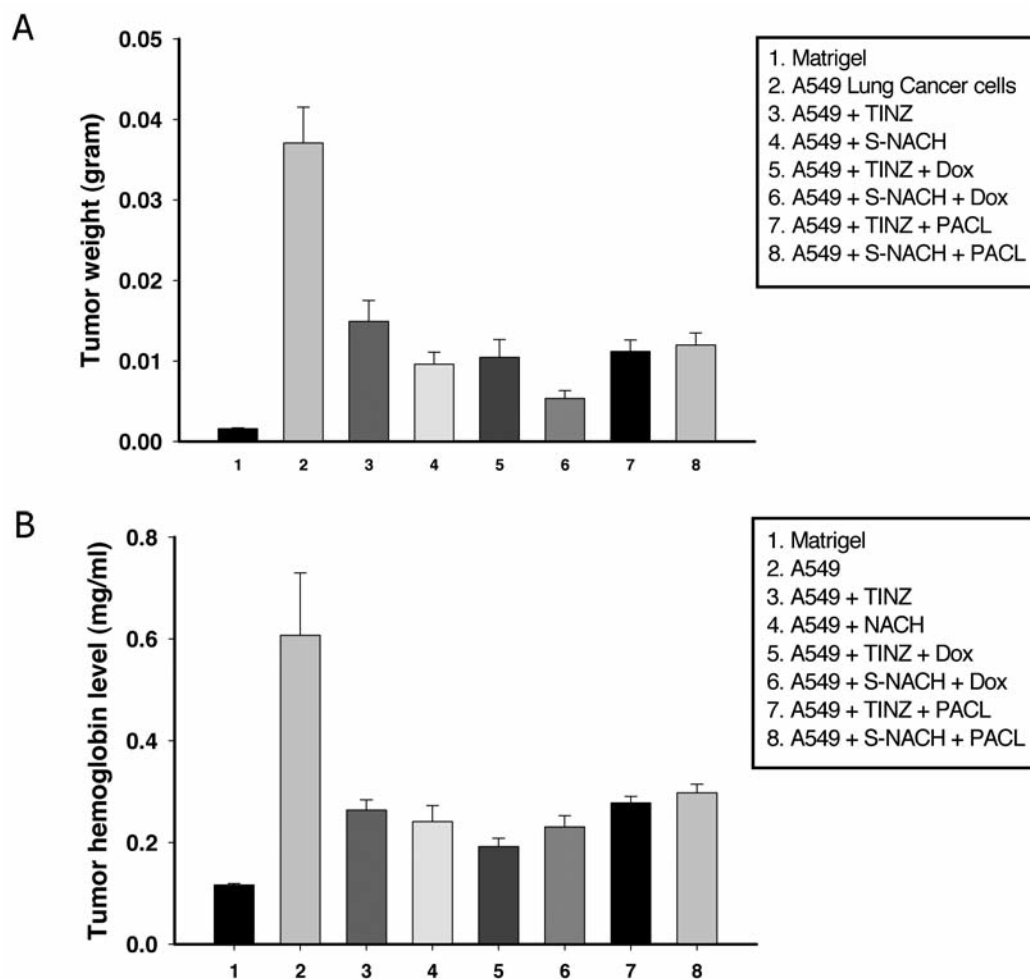


Figure 1. *TINZ* and *S-NACH* inhibit tumor growth and angiogenesis in the chick chorioallantoic membrane (CAM) model of angiogenesis and tumor growth. (A) Tumor weight (g) and (B) Hb level (mg/ml) as an index of tumor vascularity are shown. For the Dox groups, *TINZ* and *S-NACH* were given at 2.5 mg/ml (51 µg/CAM), and Dox was administered at 0.625 mg/ml (6.25 µg/CAM). For the PACL groups, PACL was given at 0.5 mg/ml (5 µg/CAM) and *TINZ* and *S-NACH* were given at 1 mg/ml (20 µg/CAM). Data are expressed as means±SEM (n=6).

at 560 nm. Chromatographic separations were carried out using a µ-Boundapak™ C₁₈ analytical column (particle size 10 µm, 125Å, 150 mm long ×3.9 mm internal diameter; Waters). The mobile phase consisted of 15 mM ammonium formate buffer (pH 3.7) containing 0.2% triethylamine and acetonitrile (35:65, v/v). The recovery of Dox was 62.1-67% from plasma, 68.6-73.6% from heart and 63.2-73.4% from lung. The limit of detectability for Dox was approximately 5 ng.

Results

TINZ and *S-NACH* inhibited tumor growth and angiogenesis of non-small cell lung carcinoma A549 implants in the CAM. The CAM tumor implant model was used for an initial assessment of the effects of LMWH and its non-anticoagulant derivative on tumor growth and angiogenesis. Administration of either *TINZ* or *S-NACH*, given separately, or in combination with Dox or PACL, significantly ($p<0.01$)

inhibited tumor growth (Figure 1A) and tumor angiogenesis (Figure 1B) following implantation of A549 cells into the CAM. Implanted tumors that were treated with the combination of *S-NACH* and Dox showed the greatest inhibition, although this inhibition did not reach the level of statistical significance as compared to *S-NACH* alone, likely because the tumor implant was effectively suppressed by treatment with LMWH or *S-NACH* alone. The fact that LMWH and *S-NACH* given alone showed major antitumor and anti-angiogenesis properties indicated that the anti-TF activity of these agents is effective in the tumor microenvironment *via* a mechanism of tumor inhibition that is complementary to that of conventional chemotherapeutic agents, which act by inhibition of tumor proliferation. These results suggested that LMWH and *S-NACH* could serve as

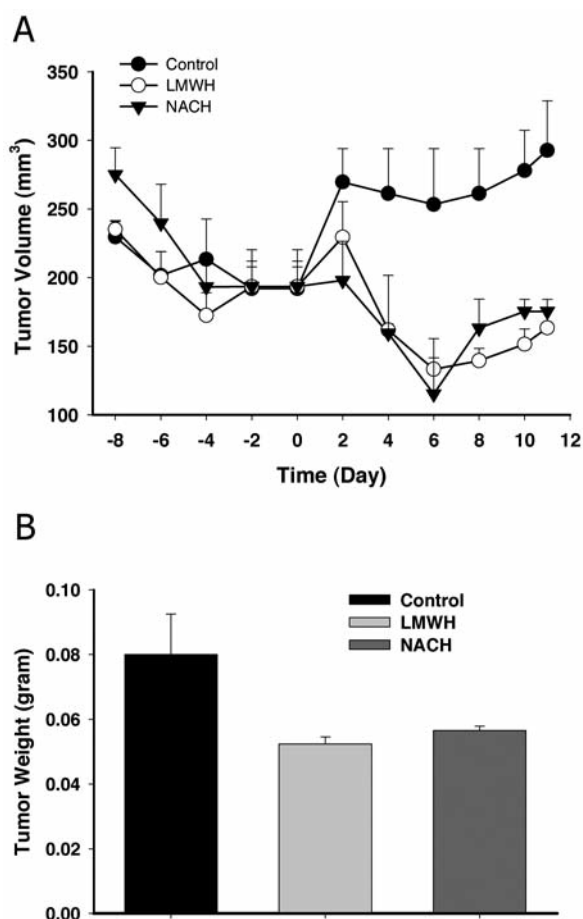


Figure 2. TINZ and S-NACH inhibit tumor growth in a mouse xenograft model of lung cancer. Tumor volume over time (A) and tumor weight at the conclusion of the experiment (B) are shown. Data represent means \pm SEM (n=4).

effective adjunct treatments.

LMWH compounds inhibit tumor growth in a mouse lung cancer xenograft model. To verify the results obtained in the CAM, A549 lung carcinoma cells (1×10^7 cells) were injected into the flanks of nude mice, resulting in the formation of tumors. Figure 2A shows the tumor growth curve (volume) for untreated *versus* TINZ- or S-NACH-treated mice. TINZ and S-NACH (each at 10 mg/kg *i.p.* daily) significantly reduced tumor growth (Figure 2A). The final tumor weights of the TINZ- and S-NACH-treated groups were significantly less than control animals (0.0800 ± 0.0125 for control *versus* 0.0524 ± 0.0022 and 0.0565 ± 0.0014 for TINZ and S-NACH, respectively). These results correlated with the results of the CAM studies (Figure 1) and showed that LMWH compounds, administered alone, effectively limit the growth of A549 lung xenografts in nude mice. Importantly, these effects on tumor growth were observed without standard

chemotherapeutic agents, providing support for the concept that this antitumor effect could potentially augment the effectiveness of chemotherapy agents, perhaps allowing for the use of lower doses of these toxic agents.

Effect of PACL alone or in combination with TINZ on human breast cancer MDA435/LCC6 xenografts. To assess the effects of combining a chemotherapeutic with a LMWH on tumor growth, the effects on PACL alone or in combination with TINZ on the growth of MDA435/LCC6 breast cancer xenografts in the nude mouse were evaluated (Figure 3). Panel 3A shows the results of 14-day treatment protocols consisting of PACL or TINZ alone, or their combination. MDA435/LCC6 breast tumor xenografts were highly responsive to PACL, such that tumor growth was completely inhibited over the course of the experimental period. TINZ alone was not inhibitory with this tumor, suggestive of possible differences in TF-dependent or TF-independent mechanisms associated with different tumor types. TINZ plus PACL treatment data points were essentially superimposable. Because PACL was so effective given alone, it was not possible to determine whether TINZ had any supplementary antitumor efficacy in this experiment. Therefore, the experiment was repeated, examining the effects of LMWH (TINZ or S-NACH) alone and in combination with PACL after discontinuation of treatment. A comparison of the growth curves of the control group and animals that were treated with PACL alone revealed that three out of the seven mice in the PACL-alone group demonstrated tumor re-growth after discontinuation of treatment, whereas all of the animals in the control group experienced tumor re-growth (Figure 3B and C, and Table I). The remaining four animals in the PACL-alone group appeared to have better responses with respect to longer complete remission (CR) and in some cases, slower tumor growth rates, defined as time to reach a preset tumor volume of 400 mm^3 , in those tumors that re-grew (Table I). The surviving animals in all groups were followed up to determine which treatment regimens were associated with prolonged survival or slowing of tumor growth. Table I summarizes the results of treatments with LMWH compounds with and without PACL. The following observations were made concerning these treatment groups: (i) expressed in terms of number of days to reach a preset tumor size of 400 mm^3 , neither of the LMWH compounds administered alone had an effect the growth of MDA435/LCC6 breast cancer xenografts as compared to the control group, consistent with the results of our initial experiment; (ii) PACL alone and in combination with each of the LMWH compounds exhibited statistically significant inhibition of tumor growth as compared with untreated controls. The absence of a median value and range for the PACL plus TINZ and PACL plus S-NACH groups indicates that the

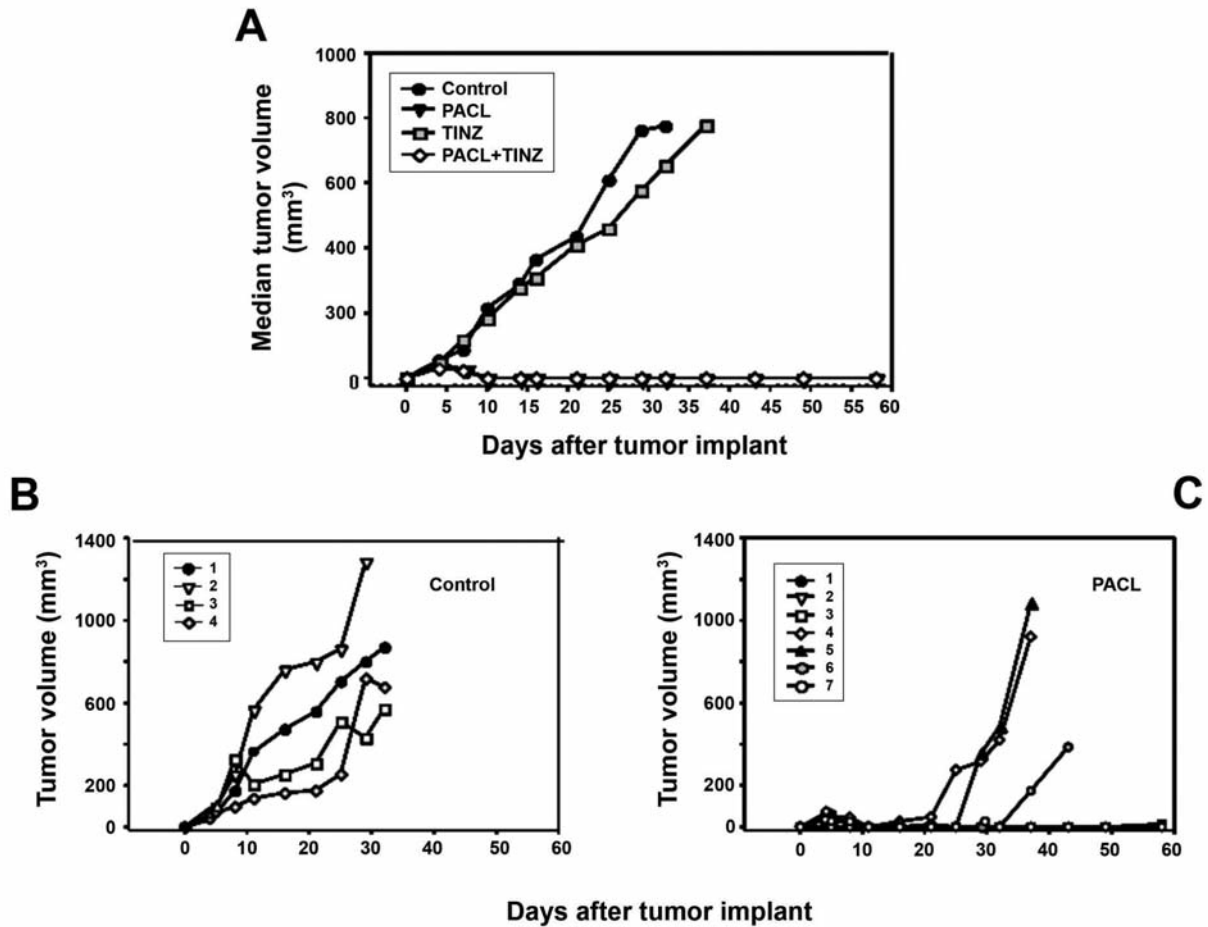


Figure 3. Antitumor efficacy of PACL with or without LMWH. (A) Results of a 14-day treatment protocol consisting of PACL or TINZ alone or their combination. Data are expressed as median tumor volume (mm^3), $n=3$. Tumor growth curves of (B) untreated (Control) and (C) PACL-treated (PACL) animals ($n=4$ and 7 , respectively) were followed after termination of treatment. The treatment protocol was as described for (A). Individual tumor growth curves are shown for each animal. Three of the seven mice in the PACL group demonstrated tumor regrowth after discontinuation of treatment on day 14.

tumors in the surviving animals did not reach 400 mm^3 within the time frame of the experiment; (iii) The combination of PACL plus S-NACH showed statistical differences in terms of time to tumor size of 400 mm^3 when compared to PACL alone, and was associated with survival times that were comparable to PACL alone, *i.e.* five out of seven animals compared to four out of seven with PACL alone. Toxicity, designated as death or $>20\%$ loss of body weight was observed in one animal in the PACL and one in the TINZ group.

Biodistribution of $[^{124}\text{I}]\text{-PACL}$ in nude mice bearing MDA435/LCC6 human breast cancer tumor xenografts. MDA435/LCC6 cells are derived from a drug-sensitive tumor and preliminary evaluations demonstrated better uptake of $[^{124}\text{I}]\text{-PACL}$ in this tumor than in the A549 lung tumor xenograft model. NCr nude mice bearing MDA435/LCC6

shoulder xenografts were divided into three treatment groups (control, TINZ and S-NACH), as per the treatment groups detailed in Table II. Animals were treated by *s.c.* injection daily for five days (10 mg/kg), and then injected with $30\text{-}60 \mu\text{Ci}$ of $[^{124}\text{I}]\text{-PACL}$ *i.v.* via tail vein injection. Animals were euthanized 24 h later, and then $[^{124}\text{I}]\text{-PACL}$ uptake in tumors and muscle was evaluated. Groups treated with LMWH compound (TINZ or S-NACH) showed greater accumulation of $[^{124}\text{I}]\text{-PACL}$ in tumors than did the controls, as expressed as $\% \text{ID/g}$ and as evaluated by tumor to muscle ratios. As shown in Table II, TINZ or S-NACH treatment resulted in a significant increase in $[^{124}\text{I}]\text{-PACL}$ uptake into tumors at 24 h when expressed as tumor to muscle ratios. Although there was variability in the $\% \text{ID/g}$ among the animals, there was a constant positive enhancement effect between controls and LMWH groups in the tumor to muscle ratio, with at least a two-fold (100%) increase in tumor to muscle ratio in each

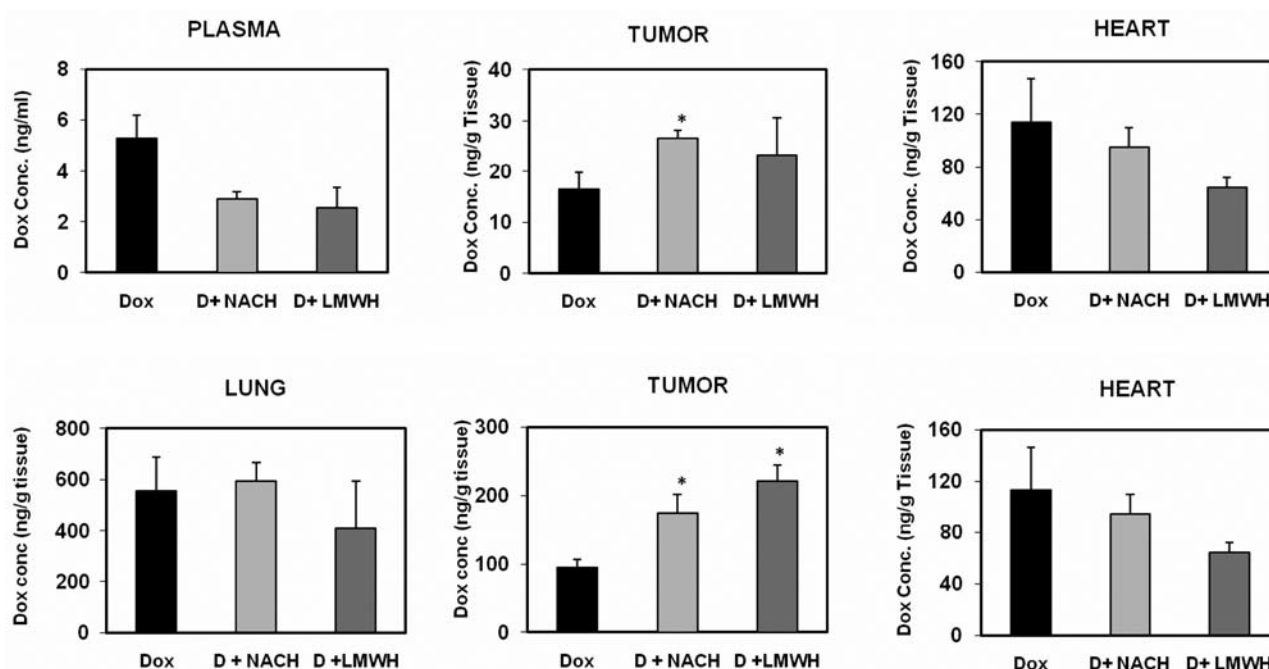


Figure 4. LMWH compounds increase uptake of chemotherapeutic agent by Dox-resistant breast tumor xenografts. Mice were pre-treated with 10 mg/kg TINZ or S-NACH for five days followed by Dox (2.5 mg/kg). Three (upper panels) or 24 h later (lower panels), animals were euthanized and tissues obtained for HPLC determination of Dox. * $p < 0.05$ versus Dox alone.

Table I. Antitumor efficacy of LMWH compounds alone and in combination with PACL in nude mice with LCC6 human breast tumor xenografts.

Treatment	Days to 400 mm ³ median (range)	P-value ^a (vs. control)	P-value ^b (vs. PACL)	Survivors/group ^c	Toxicity/group ^d
Control-a: untreated	15 (14-27)	---	<0.001	0/4	0/6
PACL	-- (30-58+)	<0.001	1	4/7	1/7
TINZ	21 (14-58+)	0.491	0.012	1/7	0/10
PACL + TINZ	--	<0.001	0.173	6/7	1/7
Control-b: untreated	25 (22-35)	--	<0.001	0/4	0/6
S-NACH	22 (15-28)	0.171	0.032	0/6	0/9
PACL + S-NACH	--	<0.001	0.0017	5/7	0/7

^aBased on comparison of each group vs. the group control using the Cox-Mantel test. ^bBased on comparison of each group vs. PACL using the Cox-Mantel test. ^cNumber of mice with tumors <400 mm³ at end of experiment/group size. ^dMice that either died or lost >20% of initial body weight.

group. This is a highly significant result in the light of the fact that the FDA criterion for a clinically meaningful effect is a 15% increase in uptake.

LMWH compounds increased uptake of Dox by MCF7 Dox-resistant tumor xenografts. The previous experiment was performed using a drug-sensitive cell line. To determine whether LMWHs increased the uptake of chemotherapeutic agent by drug-resistant tumors, the uptake of Dox by MCF7 Dox-resistant breast cancer tumor xenografts was measured using HPLC. Mice were pre-treated with 10 mg/kg of TINZ or S-NACH for five days followed by Dox (2.5 mg/kg), and

Table II. Bio-distribution of [¹²⁴I]-PACL in nude mice bearing human breast cancer LCC6 tumor xenografts[†].

Experiment	Control	Tinzaparin [†]	S-NACH
1	8.78	-	
2	2.17	32.57	8.44
3	2.15	8.28	17.83
AVG T/M [‡]	4.35	20.43	13.14

[†][¹²⁴I]-PACL uptake into tumors at 24 h is expressed as tumor to muscle ratio (T/M) average (AVG), calculated from % injected dose (ID) in tumors or muscles; n=3-4 animals. [‡]AVG T/M calculated from the results of all three experiments.

then HPLC determinations of Dox were performed 3 or 24 h after Dox treatment (Figure 4). At 3 h post-treatment, S-NACH significantly increased Dox uptake into tumors, while TINZ showed more variability than S-NACH (Figure 4, upper panels). LMWH compounds did not increase Dox levels in plasma or heart. At 24 h, both LMWH and S-NACH significantly ($p < 0.01$) selectively increased the uptake of chemotherapeutic agent by MCF7 Dox-resistant tumors by 1.5- to 2-fold, but not in heart or lung tissues (Figure 4, lower panels). These results, using an independent method of quantification, were strikingly similar to the findings obtained with [^{124}I]-PACL. In particular, these findings were obtained using a drug-sensitive tumor cell line. These results are particularly significant in light of the fact that Dox is associated with serious cardiotoxicity.

Discussion

The experimental data in this report provide mechanistic insights and additional support for the use of LMWH for treatment of cancer patients. Studies performed in three separate *in vivo* tumor model systems demonstrated that: (i) LMWH administered alone or in the presence of chemotherapeutic agents significantly inhibits human A549 lung carcinoma growth and angiogenesis in the CAM tumor implant (Figure 1) and mouse xenograft models (Figure 2) and (ii) *in vivo* experiments performed in a human MDA435/LCC6 PACL-sensitive breast tumor xenograft model showed that PACL inhibition of tumor growth obscured possible supplementary effects of LMWH over a short-duration course of therapy (14 days). However, in the follow-up experiments, while some PACL-treated mice showed tumor re-growth after discontinuation of the 14-day treatment protocol, the combination of a LMWH compound provided an advantage in terms of prolonged survival and/or slowing of tumor growth (Table I). Regimens that included a LMWH were associated with reduced tumor growth rate and in some cases, improved survival rates. These results correlate with clinical studies demonstrating survival advantage in certain subgroups of patients treated with subcutaneous heparin (28) or LMWH therapy (24) that were seen for months and years after the period of active administration.

Importantly, this study demonstrated for the first time that LMWH administration significantly increases uptake of chemotherapeutic agents into tumors. Two independent means of quantification, gamma counting of radiolabeled PACL in drug-sensitive MDA435/LCC6 tumors (Table II) and HPLC evaluation of Dox in Dox-resistant MCF-7 tumors (Figure 4), confirmed that LMWH greatly improves the uptake of these agents into tumors. In the case of Dox, LMWH selectively increased tumor uptake while reducing the uptake into heart, a site of significant toxicity with this drug. Both the conventional

LMWH TINZ and S-NACH were effective in influencing drug uptake and antitumor efficacy. This is important because NACHs have minimal effects on hemostasis (bleeding time, aPTT and PT values), and their use may represent an improved treatment profile for possible long-term usage of these compounds for clinical treatment, if required.

There are several possible mechanisms that might be involved in the augmentation of tumor uptake of chemotherapy agents. Glycosaminoglycans are complex multifunctional compounds that exhibit an array of biological effects that include inhibition of P- and L-selectin-dependent processes, and interference with growth factor-initiated processes (29, 30). Unfractionated heparins have been shown to impact drug resistance *via* effects on P-glycoprotein and associated pump activity *in vitro* in the MDA-231 breast cancer cell line (31), one of the principal mechanisms of drug resistance (reviewed in (32)). Furthermore, localized production of TFPI in the tumor vasculature might minimize the accumulation of fibrin and plasma proteins in the tumor microenvironment, reducing the build-up of interstitial pressure that impedes delivery of chemotherapeutic agents to the interior of the tumor. Future studies will investigate whether the ability of LMWH to increase chemotherapeutic uptake is a more generalized phenomenon, occurring with other tumor-chemotherapeutic drug combinations in addition to those reported in this study. Other studies will focus on defining TF-dependent and -independent mechanisms of action of LMWH on chemotherapeutic uptake and antitumor efficacy. Protocols utilizing adjuvant or neoadjuvant therapy with LMWH might lead to reduction in the doses of chemotherapy required because of more efficient uptake by tumors.

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References

- 1 Levine M and Hirsh J: The diagnosis and treatment of thrombosis in the cancer patient. *Semin Oncol* 17: 160-171, 1990.
- 2 Mousa SA: Low-molecular-weight heparin in thrombosis and cancer. *Semin Thromb Hemost* 30: 25-30, 2004.
- 3 Sproul EE: Carcinoma and venous thrombosis: the frequency of association of carcinoma in the body or tail of the pancreas with multiple venous thromboses. *Am J Cancer* 34: 566-585, 1938.
- 4 Mousa SA: Anti-thrombotics in thrombosis and cancer. *Future Oncol* 1: 395-403, 2005.
- 5 Nagy JA, Brown LF, Senger DR, Lanir N, Van de Water L, Dvorak AM and Dvorak HF: Pathogenesis of tumor stroma generation: a critical role for leaky blood vessels and fibrin deposition. *Biochim Biophys Acta* 948: 305-326, 1989.
- 6 Jain RK: Understanding barriers to drug delivery: high resolution *in vivo* imaging is key. *Clin Cancer Res* 5: 1605-1606, 1999.

- 7 Jain RK: Normalizing tumor vasculature with anti-angiogenic therapy: a new paradigm for combination therapy. *Nat Med* 7: 987-989, 2001.
- 8 McDonald DM and Baluk P: Significance of blood vessel leakiness in cancer. *Cancer Res* 62: 5381-5385, 2002.
- 9 Broze GJ: The tissue factor pathway of coagulation. *In: Thrombosis and Hemorrhage*. Loscalzo J and Schafer AI (eds.). Baltimore, William and Wilkins, pp. 77-104, 1998.
- 10 Carmeliet P, Mackman N, Moons L, Luther T, Gressens P, Van Vlaenderen I, Demunck H, Kasper M, Breier G, Evrard P, Muller M, Risau W, Edgington T and Collen D: Role of tissue factor in embryonic blood vessel development. *Nature* 383: 73-75, 1996.
- 11 Chen J, Bierhaus A, Schiekofer S, Andrassy M, Chen B, Stern DM and Nawroth PP: Tissue factor – a receptor involved in the control of cellular properties, including angiogenesis. *Thromb Haemost* 86: 334-345, 2001.
- 12 Kakkar AK, Lemoine NR, Scully MF, Tebbutt S and Williamson RC: Tissue factor expression correlates with histological grade in human pancreatic cancer. *Br J Surg* 82: 1101-1104, 1995.
- 13 Belting M, Dorrell MI, Sandgren S, Aguilar E, Ahamed J, Dorfleutner A, Carmeliet P, Mueller BM, Friedlander M and Ruf W: Regulation of angiogenesis by tissue factor cytoplasmic domain signaling. *Nat Med* 10: 502-509, 2004.
- 14 Bora PS, Hu Z, Tezel TH, Sohn JH, Kang SG, Cruz JM, Bora NS, Garen A and Kaplan HJ: Immunotherapy for choroidal neovascularization in a laser-induced mouse model simulating exudative (wet) macular degeneration. *Proc Natl Acad Sci USA*, 100: 2679-2684, 2003.
- 15 Mousa S: Tissue factor pathway inhibitor in thrombosis and beyond. *In: Anticoagulants, Antiplatelets, and Thrombolytics*. Mousa S (ed.). Totowa, Humana Press Inc., pp. 133-155, 2004.
- 16 Mousa SA, Linhardt R, Francis JL and Amirkhosravi A: Anti-metastatic effect of a non-anticoagulant low molecular weight heparin *versus* the standard low molecular weight heparin, enoxaparin. *Thromb Haemost* 96: 816-821, 2006.
- 17 Mousa SA and Mohamed S: Anti-angiogenic mechanisms and efficacy of the low molecular weight heparin, tinzaparin: anti-cancer efficacy. *Oncol Rep* 12: 683-688, 2004.
- 18 Mousa SA and Mohamed S: Inhibition of endothelial cell tube formation by the low molecular weight heparin, tinzaparin, is mediated by tissue factor pathway inhibitor. *Thromb Haemost* 92: 627-633, 2004.
- 19 Lupu C, Poulsen E, Roquefeuil S, Westmuckett AD, Kakkar VV and Lupu F: Cellular effects of heparin on the production and release of tissue factor pathway inhibitor in human endothelial cells in culture. *Arterioscler Thromb Vasc Biol* 19: 2251-2262, 1999.
- 20 Lemoine NR: Antithrombotic therapy in cancer. *J Clin Oncol* 23: 2119-2120, 2005.
- 21 Kakkar AK, Levine MN, Kadziola Z, Lemoine NR, Low V, Patel HK, Rustin G, Thomas M, Quigley M and Williamson RC: Low molecular weight heparin, therapy with dalteparin, and survival in advanced cancer: the fragmin advanced malignancy outcome study (FAMOUS). *J Clin Oncol* 22: 1944-1948, 2004.
- 22 Altinbas M, Coskun HS, Er O, Ozkan M, Eser B, Unal A, Cetin M and Soyuer S: A randomized clinical trial of combination chemotherapy with and without low molecular weight heparin in small cell lung cancer. *J Thromb Haemost* 2: 1266-1271, 2004.
- 23 Lee AY, Rickles FR, Julian JA, Gent M, Baker RI, Bowden C, Kakkar AK, Prins M and Levine MN: Randomized comparison of low molecular weight heparin and coumarin derivatives on the survival of patients with cancer and venous thromboembolism. *J Clin Oncol* 23: 2123-2129, 2005.
- 24 Klerk CP, Smorenburg SM, Otten HM, Lensing AW, Prins MH, Piovella F, Prandoni P, Bos MM, Richel DJ, van Tienhoven G and Buller HR: The effect of low molecular weight heparin on survival in patients with advanced malignancy. *J Clin Oncol* 23: 2130-2135, 2005.
- 25 El-Naggar MM and Mousa SA: Patent No. US 6,908,907 B2, issued June 21, 2005, and Mousa SA, Patent Pending: U.S. Provisional Application 60/411,851, filed September 20, 2002 and full US patent, 10, 667,216, September 19, 2003.
- 26 Vredenburg MR, Ojima I, Veith J, Pera P, Kee K, Cabral F, Sharma A, Kanter P, Greco WR and Bernacki RJ: Effects of orally active taxanes on P-glycoprotein modulation and colon and breast carcinoma drug resistance. *J Natl Cancer Inst* 93: 1234-1245, 2001.
- 27 Mousa SS and Mousa SA: Effect of resveratrol on angiogenesis and platelet/fibrin-accelerated tumor growth in the chick chorioallantoic membrane model. *Nutr Cancer* 52: 59-65, 2005.
- 28 Lebeau B, Chastang C, Brechot JM, Capron F, Dautzenberg B, Delaisements C, Mornet M, Brun J, Hurdebourcq JP and Lemarie E: Subcutaneous heparin treatment increases survival in small cell lung cancer. 'Petites Cellules' Group. *Cancer* 74: 38-45, 1994.
- 29 Smorenburg SM and Van Noorden CJ: The complex effects of heparins on cancer progression and metastasis in experimental studies. *Pharmacol Rev* 53: 93-105, 2001.
- 30 Stevenson JL, Varki A and Borsig L: Heparin attenuates metastasis mainly due to inhibition of P- and L-selectin, but non-anticoagulant heparins can have additional effects. *Thromb Res* 120(Suppl 2): S107-S111, 2007.
- 31 Angelini A, Di Febbo C, Ciofani G, Di Nisio M, Baccante G, Di Ilio C, Cucurullo F and Porreca E: Inhibition of P-glycoprotein-mediated multidrug resistance by unfractionated heparin: a new potential chemosensitizer for cancer therapy. *Cancer Biol Ther* 4: 313-317, 2005.
- 32 Luqmani YA: Mechanisms of drug resistance in cancer chemotherapy. *Med Princ Pract* 14(Suppl 1): 35-48, 2005.

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