Inhibition of PAR-1 Receptor Signaling by Enoxaparin Reduces Cell Proliferation and Migration in A549 Cells

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Abstract. *Background/Aim:* Low-molecular weight heparins (LMWHs) may possess putative antitumoral properties; however, the underlying mechanism(s) remains elusive. We evaluated the antiproliferative and antimigratory effects of enoxaparin (a LMWH) in lung adenocarcinoma A549 cells, and assessed the possible mechanism involved, and the effect on doxorubicin's efficacy. *Materials and Methods:* Proliferation and migration were evaluated using BrdU and transwell assays, respectively. Immunoblotting was used to measure PAR-1, PAR-2, MMP-2, ERK1/2 and Akt proteins. Apoptosis and cell cycle studies examined the combined effect of enoxaparin and doxorubicin. *Results:* Enoxaparin inhibited A549 cell proliferation and migration. Following PAR-1 gene knock down, enoxaparin's effect on A549 cell proliferation was diminished compared to scrambled siRNA. Our experiments verified that enoxaparin-mediated down-regulation of MAPK and PI3K, reduced MMP-2 expression and inhibited A549 cell migration. Additionally, enoxaparin increased doxorubicin's efficacy by enhancing apoptosis, while no effect on cell-cycle progression was observed. *Conclusion:* Results suggest that the anticancer activity of enoxaparin in A549 cells was mediated by the interference of two major PAR-1 downstream signaling pathways, MAPK/ERK and PI3K/Akt, which in turn inhibit proliferation and migration. Therefore, enoxaparin may be promising as an adjunct to traditional chemotherapy for lung cancer and warrants further investigation.

Lung cancer is one of the most fatal malignancies in the United States with a higher mortality rate than breast, prostate and colon cancer combined (1). However, lung cancer is a relatively under-studied malignancy with respect to treatment options and improving its prognosis and survival rate. There has been a growing interest in the enhanced clinical outcomes seen among cancer patients taking concomitant low-molecular weight heparin (LMWH) for the management of venous thromboembolism (VTE) alongside standard chemotherapeutic treatments. LMWHs are short chains of polysaccharides, approximately 2-8 kDa, prepared by depolymerization of unfractionated heparin (2). Until recently, LMWHs have been the anticoagulants of choice in cancer patients with acute thrombosis (3). However, data from recent randomized clinical trials comparing two of the new direct oral anticoagulants (DOACs; edoxaban or rivaroxaban) *versus* LMWHs have shown DOACs to be at least non-inferior to the LMWHs in the treatment of cancer-associated VTE, and this has led to a guidance statements recommending the use of these newer agents only in cancer patients with low bleeding risk (4). Nonetheless, there is controversial data concerning LMWHs' ability to suppress cancer progression, which has hindered their clinical use outside the treatment of VTE, where these agents could potentially provide additional benefit in the form of increased cancer survival and improved clinical outcomes. For this reason, it was imperative to clearly understand the underlying mechanism(s) of the prospective anticancer activities of LMWHs that have been observed in many clinical and preclinical studies (5-8).

Cancer patients are 5-7 times more susceptible to VTE compared to non-cancer patients (9). Investigators have shown a significant association between VTE incidence and higher malignancy (10). This suggests a strong correlation between the hemostatic system and cancer pathophysiology. Although the connection between the two is not fully understood, studies have demonstrated a two-sided interaction, supporting the notion that activation of the blood coagulation pathways may accelerate cancer progression and

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contrariwise. On one hand, tumor cells and their products have the ability to activate the coagulation system and contribute to the acquired hypercoagulable state (11, 12). On the other hand, evidence has demonstrated that elevated hemostatic proteases can positively impact tumor progression (10, 13). There are several different molecular mechanisms by which these hemostatic components influence tumor biology. Nevertheless, activation of the protease-activated receptors (PARs) appears to be an important mechanism for tumor development and progression (10, 13-15).

The PARs belong to a subfamily of the G-protein coupled receptors (GPCRs) and they carry their own ligand that is activated by a serine protease. Activation of PARs trigger different downstream signaling pathways resulting in a variety of cellular responses, including thrombosis, hemostasis, cell survival, proliferation and inflammation (16). Studies have also demonstrated that PARs play a pivotal role in cancer pathophysiology (14, 17). Of the four PAR isoforms, PAR-1 and PAR-2 are up-regulated in many human cancers, such as lung, liver, breast, colon, prostate and melanoma (18). Additionally, investigators have shown a strong association between tumor aggressiveness and expression levels of PARs (19-24). As a result of the cancer-associated hypercoagulation, PARs activation by the elevated hemostatic proteases have shown to induce cell growth, proliferation, angiogenesis and metastasis, which subsequently impact tumorigenesis. Based on these findings, we hypothesized that anticoagulant therapies that target and limit those proteases, such as the LMWH enoxaparin, could be a helpful clinical tool to reduce cancer progression and improve prognosis. Clinical studies have shown that LMWHs used for VTE have significantly improved survival rates and clinical outcomes in many malignancies independently of their anticoagulant function (6, 25). Furthermore, there are appealing preclinical studies showing that LMWHs exert antitumor effects *via* multiple mechanisms (7, 8). An *in vitro* study has demonstrated that enoxaparin significantly decreases cell growth, but not cell invasion in primary cell cultures of high-grade gliomas. The antiproliferative effect was more prominent in cultures with higher PAR-1 expression, although no conclusive relationship between PAR-1 and enoxaparin's activity was made (26). Enoxaparin has also been shown to significantly inhibit thrombin-induced osteosarcoma proliferation both *in vitro* and *in vivo* (27). Additionally, both enoxaparin and dalteparin can inhibit migration of patients-derived vascular smooth muscle cells (28). Moreover, enoxaparin reduced lung metastasis by 70% in an experimental mouse model (29).

With the extremely complicated and interactive relationship between the coagulation system and cancer, as well as the unclear mechanisms underlying LMWHs' antitumoral effects, we aimed to investigate the antineoplastic characteristics of enoxaparin, more specifically its antiproliferative and antimigratory effects in the A549 lung

adenocarcinoma cell line and delineate the potential molecular mechanisms involved. Furthermore, we assessed enoxaparin's ability to improve doxorubicin's efficacy in A549 and the mechanism(s) involved.

Materials and Methods

Cell culture and reagents. A549 cell line was purchased from ATCC (Manassas, VA, USA) and maintained in complete Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin antibiotics that were all obtained from ATCC (Manassas, VA, USA). Cells were kept at 37°C in a 5% CO₂ incubator. Enoxaparin and doxorubicin were obtained from Cardinal Health (Dublin, OH, USA). Genistein and staurosporine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human α -thrombin was purchased from Haematologic Technologies (Essex, VT, USA). Primary antibodies against PAR-1, PAR-2 and thrombin were obtained from Abcam (Cambridge, MA, USA). Antibodies against ERK1/2, p-ERK1/2, Akt, and p-Akt were purchased from Cell Signaling (Danvers, MA, USA). Secondary antibody was obtained from EMD Millipore (Temecula, CA, USA). Gene probes for PAR-1, MMP-1, MMP-2, MMP-3, MMP-9 as well as PAR-1 and scrambled siRNAs were purchased from ThermoFisher (Rockford, IL, USA).

Bromodeoxyuridine (BrdU) cell proliferation assay. Cells were seeded in a 96-well plate (5×10³/well) using complete DMEM and were allowed to attach overnight. Media was then replaced with fresh DMEM supplemented with 5% FBS and was subsequently treated with enoxaparin or doxorubicin for 24 h. For PAR-1 activation with α -thrombin, cells were seeded as previously described and were allowed to attach for 4 h, then media was replaced with fresh serum-free DMEM and cells were subsequently treated with α -thrombin for 24 h. BrdU labeling solution was added 1 h after the treatments. An ELISA BrdU was performed as per the manufacturer's (Roche Diagnostics; Florham Park, NJ, USA) protocol. Absorbance was measured at 450 nm using microplate reader SYNERGY/HT.

Migration assay. Cell migration was studied using 8 μ m pore 24-well transwell chambers purchased from ThermoFisher. Cell suspension (3×10⁴) in serum-free media containing enoxaparin was added inside the inserts. Complete DMEM was added to the lower chambers as the chemoattractant. The migration plate was incubated at 37°C for 48 h. Media was removed and the inserts system were immediately transferred to a clean 24-well plate containing 500 μ l of Calcein-AM and was incubated at 37°C for 1 h. Fluorescence was measured at 494 nm/517 nm from the bottom of the well.

Apoptosis assay. Cells were seeded in a 6-well plate (3×10⁵/well) using complete DMEM and allowed to attach overnight. Media was replaced with DMEM containing 5% FBS plus enoxaparin and doxorubicin alone or combined and incubated for 24 h. Staurosporine (STS, 4 μ M), a commonly used protein kinase inhibitor and activator of apoptosis, was used as positive control (30, 31). STS was dissolved in DMSO and diluted to the final concentration (4 μ M) to contain 0.4% DMSO. A control well for the 0.4% DMSO solvent was tested to ensure it did not induce apoptosis. Media was collected, cells were trypsinized and resuspended in 500 μ l binding buffer. All steps were carried out on

ice. Annexin V-FITC (1 μ l) and propidium iodide (PI) (1 μ l) were added to cell suspensions and were incubated for 15 minutes in the dark at room temperature (RT). Samples were processed by fluorescence-activated cell sorting (FACS) flow cytometry from Millipore (Danvers, MA, USA).

Cell cycle assay. Cells were seeded in a 6-well plate (3×10^5 /well) using complete DMEM and allowed to attach overnight. Cells were serum-starved for 24 hours to synchronize them at G₀ phase. Media were replaced with DMEM containing 5% FBS plus enoxaparin and/or doxorubicin and were incubated for 24 h. Genistein (30 μ M), a potent protein tyrosine kinase (PTK) inhibitor, was used as positive control to induce G₂/M phase arrest in non-small cell lung cancer (32). Genistein was dissolved in ethanol and diluted to the final concentration (30 μ M) to contain 0.3% ethanol. A control well for the 0.3% ethanol solvent was tested to ensure it has no effect on cell cycle progression. Cells were harvested, washed with PBS and fixed with 70% ethanol for 30 min at 4°C. Cells were re-suspended in a solution containing PBS, RNase and PI (250:1:5), then incubated at 37°C for 30 min. Cell cycle analysis was performed using FACS flow cytometry (Millipore).

Western blotting assay. Protein expression of thrombin, PAR-1, PAR-2 and MMP-2 were measured using Western Blot. A549 and HepG2 cells were seeded in T-75 flask using DMEM media supplemented with 10% FBS and 1% Pen Strep and allowed to grow to 80% confluence. To investigate the effect of enoxaparin on the activation and phosphorylation of the MAPK (ERK1/2) as well as the PI3K (Akt), A549 cells were seeded in T-25 flask (7×10^5 cells/5 mL/flask) using DMEM media supplemented with 10% FBS and 1% Pen Strep and were allowed to attach overnight. The next day the media was replaced with fresh DMEM supplemented with 5% FBS and 1% Pen Strep, and cells were subsequently treated with final concentrations of enoxaparin for 24 h. Cells were detached using trypsin and proteins were extracted using RIPA buffer and were then reduced with Laemmli buffer. To investigate the effect of enoxaparin on the activation of MAPK (ERK1/2) and PI3K (Akt), at the end of incubation period media was removed and cells were washed and subsequently scraped using blue loading buffer prepared as in the manufacturer's (Cell Signaling) protocol (Danvers, MA, USA). Samples were separated in 10% tris-glycine gels and were then transferred to nitrocellulose membranes. Membranes were blocked with 5% BSA (ERK1/2 and Akt) or 5% non-fat milk (thrombin, PAR-1, PAR-2 and MMP-2) for 1 hour. Membranes were incubated overnight with primary antibodies against thrombin (1:1,000), PAR1 (1:1,000), PAR-2 (1:5,000), MMP-2 (1:500), ERK1/2 (1:1,000), p-ERK1/2 (1:2,000), Akt (1:1,000) and p-Akt (1:2,000). Blots were incubated with secondary antibody (1:5000) for 1 hour and were visualized using LI-COR C-DiGit scanner (Lincoln, NE, USA). Band density was analyzed using Image Studio Digits Ver4.0 software (LI-COR, Lincoln, NE, USA). All protein expressions were normalized to beta-actin.

PAR-1 knockdown and PCR gene expression assay. A549 cells were seeded in a 6-well plate as previously mentioned. Opti-MEM medium was used to dilute lipofectamine reagent (9 μ l:150 μ l) and PAR-1 siRNA (3 μ l:150 μ l). Diluted siRNA and Lipofectamine were mixed (1:1) and added to wells with a final concentration of 25 nM. Cells were incubated 3 days and qPCR was performed to determine knockdown efficiency. Total RNA was extracted from samples with trizol, chloroform, isopropanol then 75% ethanol. Optical density

(260/280) between 1.8-2.0 is considered pure and all RNA samples used were found to be in this range. Total RNA was reverse transcribed into cDNA. RT-PCR was performed using Applied Biosystems StepOnePlus from ThermoFisher Scientific (Waltham, MA, USA). All mRNA expressions were normalized to GAPDH. Relative fold change of gene expression was calculated using the comparative CT ($\Delta\Delta C_T$) method.

Statistical analysis. Results are presented as mean $\pm$ standard deviation (SD) for minimum of three independent experiments. Statistical differences were measured by student's *t*-test for two groups, or a one-way ANOVA for multiple groups, followed by Tukey's test, using GraphPad Prism7 (San Diego, CA, USA). To be considered statistically significant, *p*-Value was <0.05.

Results

A549 cell proliferation was evaluated after treatment with various concentrations of enoxaparin (0.22-66 μ M) in a reduced serum media (5% FBS). Results demonstrated that a 24-hour enoxaparin treatment at 22, 44, and 66 μ M significantly inhibited cell proliferation by 10, 13, and 15%, respectively, compared to control (Figure 1A). The transwell migration assay was carried out to determine the potential inhibitory effect of enoxaparin on cell migration of the A549 cells. Results showed that pretreatment with enoxaparin (22 μ M) for 48 hours slightly reduced cell migration and treatment with higher concentrations (44 and 66 μ M) significantly reduced *in vitro* cell migration, a surrogate marker for metastasis, of the pulmonary adenocarcinoma A549 cells by 15 and 36%, respectively, compared to negative control (Figure 1B).

Since A549 cell line expresses high levels of PAR-1 receptor while it only expresses very low levels of PAR-2 (Figure 2), we investigated the functional significance of abundant PAR-1 expression in mediating A549 cell proliferation. Data showed that treatment with α -thrombin, a known activator of PAR-1, resulted in significant induction of cell growth by 7, 13 and 20% increase for the 2.5, 5 and 10 nM, respectively (Figure 3). To further confirm the connection between PAR-1 and A549 cell proliferation, PAR-1 gene was silenced by siRNA. qPCR analysis demonstrated that siRNA post-transfection significantly reduced PAR-1 mRNA by 92% compared to control (data not shown). To examine whether PAR-1 knockdown would affect the proliferative behavior of A549, cell proliferation with and without PAR-1 siRNA transfection was measured. We found that in comparison to control, wells transfected with PAR-1 siRNA showed significant decrease in cell proliferation by almost 45% compared to scrambled siRNA (Figure 4A). These results suggest that PAR-1 play a vital role in mediating cell division and proliferation of the pulmonary adenocarcinoma A549 cells. To link the inhibitory capability of enoxaparin to PAR-1, we examined cell proliferation of the A549 cells following PAR-1 knockdown with and without enoxaparin treatments.

Figure 1. Inhibition of A549 cell proliferation and migration by enoxaparin. (A) BrdU ELISA assay was performed following a 24-h treatment of A549 with enoxaparin (0.22-66 μ M). Absorbance was measured at 450 nm using microplate reader SYNERGY/HT. Data are presented as a mean $\pm$ S.D. of six independent experiments. * p <0.05 ** p <0.001 versus control group or versus respective treated groups (ANOVA). (B) Transwell migration assay was conducted following treatment with enoxaparin (22, 44 and 66 μ M) for 48 hours. Fluorescence was measured at 494 nm/517 nm from bottom using a microplate reader SYNERGY/HT. Data are presented as a mean $\pm$ S.D. of four independent experiments. * p <0.05 *** p <0.0001 versus control group or versus respective treated groups (ANOVA).

We used enoxaparin concentrations that showed a significant inhibition of proliferation in our study. Our results revealed that PAR-1 knockdown not only reduced cell proliferation compared to non-siRNA treated control, but it also abolished the enoxaparin inhibitory effect under all concentrations when compared to PAR-1 siRNA treated control (Figure 4B). These results suggest that the antiproliferative effect of enoxaparin is mediated through PAR-1.

To additionally support the hypothesis that enoxaparin-induced inhibition of A549 cell proliferation is mediated through a PAR-1 dependent mechanism, we examined enoxaparin's effect on PAR-1 downstream signaling cascades, specifically on the MAPK/ERK and PI3K/Akt protein expression profiles. Our data demonstrated that enoxaparin inhibited the activation of the MAPK downstream signaling protein ERK1/2, as evident by a decreased ratio of p-ERK1/2: total ERK1/2 (Figures 5A and B). Treatment with enoxaparin (66 μ M) down-regulated p-ERK1/2 expression by more than 50%. Moreover, treatment with enoxaparin (22 μ M 44 and 66 μ M) down-regulated p-Ak by about 25, 70 and 80%, respectively (Figures 5C and D). These results imply that both inhibition and interference of A549 cell proliferation by enoxaparin occur through a PAR-1 dependent mechanism.

Matrix metalloproteinases (MMPs) are enzymes that degrade the extracellular matrix (ECM) and promote invasion and metastasis in malignant tumors. Several studies

have indicated that at the transcriptional level, MMPs expression is regulated *via* the MAPK or PI3K pathways (33-36). To investigate the ability of enoxaparin to modulate MMPs expression as a consequence of suppressing the MAPK and PI3K pathways, different MMPs were measured after cells were pretreated with concentrations of enoxaparin, which significantly reduced cell migration. Enoxaparin significantly reduced MMP-2 mRNA by 65 and 75% for the 44 and 66 μ M, respectively (Figure 6A). However, no effect was observed on MMP-1 and MMP-9 gene expression by enoxaparin (data not shown). Moreover, MMP-3 could not be detected in A549 cell line. Results also revealed that enoxaparin down-regulated MMP-2 protein levels more than 50% at 66 μ M (Figures 6B and C). Our results suggest that *in vitro* suppression of A549 cell migration by enoxaparin possibly occurred as a result of enoxaparin-mediated down-regulation of MMP-2 expression *via* the inhibition of MAPK and PI3K pathways.

To assess the ability of enoxaparin to increase doxorubicin efficacy, the low concentration of 100 nM doxorubicin was selected, based on its ability to inhibit proliferation without masking enoxaparin's effect (data not shown), and 44 μ M of enoxaparin was selected due to its ability to significantly inhibit both cell proliferation and migration. Our data showed that the combination of doxorubicin plus enoxaparin reduced cell proliferation of A549 by approximately 25% in

Figure 2. Semiquantitative expression levels of PAR-1 and PAR-2 proteins in A549. Western blot experiments were conducted to assess expression levels of PAR-1 (A and C) and PAR-2 (B and D) in A549 and HepG2 cells. In the data shown, A and B are graphical representation of three independent experiments, while C and D are blots of one representative experiment. HepG2 cell line was used as positive control. Protein expression was normalized to β -actin. Data are presented as a mean $\pm$ S.D. of three independent experiments. * $p < 0.0001$ versus positive control group (Student's *t*-test).

comparison to control. However, when compared to doxorubicin alone, the combined treatment inhibited cell growth by an average of 15% more (Figure 7). These results suggest that enoxaparin has the ability to increase the efficacy of the chemo-agent doxorubicin in A549 cells.

To explore the mechanism of doxorubicin improved inhibition of A549 cell proliferation by enoxaparin, we investigated the effect of the combination treatment on apoptosis. Administration of enoxaparin induced neither early nor late apoptosis (Figure 8). In contrast, the positive control staurosporine significantly promoted apoptosis by 43% (Figure

8B). Doxorubicin induced slight apoptosis by 6% in total (Figure 8B). Doxorubicin in combination with enoxaparin (Figure 8B) significantly increased the percentage of early and late apoptotic cells by 11 and 4%, respectively, compared to 4 and 2% for doxorubicin monotherapy. These data suggest that enoxaparin enhanced doxorubicin's inhibition of A549 cell proliferation through induction of apoptosis.

Furthermore, we analyzed the cell cycle status of the A549 with enoxaparin, doxorubicin and their combination treatment. Incubation with doxorubicin (Figure 9) altered the cell cycle pattern with significant increase in the number of

Figure 3. Effect of PAR-1 activation by thrombin on A549 cell proliferation. BrdU ELISA assay was carried out after treatment with α -thrombin (2.5, 5 and 10 nM) for 24 h in A549 cells. Absorbance was measured at 450 nm using microplate reader SYNERGY/HT. Data are presented as a mean $\pm$ S.D. of three independent experiments. * $p < 0.0001$ versus control group or versus respective treated groups (ANOVA).

cells in G₀/G₁ phases (48.5%) and decrease in S and G₂/M phase (13.9% and 37.6%). Cell cycle distribution following 44 μ M enoxaparin treatment was not altered compared to control. In comparison to doxorubicin alone, very similar cell distribution was observed for doxorubicin and enoxaparin co-treatment (Figure 9), suggesting that doxorubicin-induced cell cycle alteration was not affected by enoxaparin treatment.

Our data verified that in addition to its anticoagulant function the LMWH enoxaparin is capable of inhibiting two key processes of tumorigenesis, proliferation and migration, in the lung adenocarcinoma A549 cells by the interference of two major PAR-1 downstream signaling pathways, the MAPK/ERK and PI3K/Akt (Figure 10).

Discussion

Management of VTE with anticoagulant therapies in cancer patients is a challenging task, yet it is essential to decrease morbidity and mortality of patients suffering from tumor-associated thromboembolism. However, it is important to mention that the detrimental effect of VTE on the survival rate among cancer patients is not solely as a result of the thromboembolic event itself, but also of a more aggressive tumor phenotype. LMWHs have been shown to improve the survival rate of small cell lung cancer patients and those with advanced stage tumors who have a more favorable prognosis (37).

Researchers have been studying several different LMWHs' impact on lung cancer progression *in vitro*. Chen *et al.*, have found that dalteparin can decrease cell viability and induce both G₁ phase arrest and apoptosis in A549 cells, effects that were due to increased levels of p21 and p27 (38). Another study using nadroparin, has demonstrated a decrease in A549 cell proliferation by inducing G₂/M phase arrest due to inhibition of cyclin B1, Cdk1, and Cdc25C (39). In the melanoma (WM-266-4) and pancreatic (AsPC-1) cancer cell lines, Featherby *et al.*, have shown that both tinzaparin and dalteparin suppresses tumor growth, invasion and vascularization (40). Also, Arab *et al.*, have revealed a concentration- and time-dependent reduction in A549 cell proliferation by down-regulating CD44 and c-Myc expression when treated with enoxaparin (41).

In agreement with other preclinical findings for the potential anti-tumoral activities of LMWHs, the current study validated that enoxaparin can modulate A549 cell proliferation and migration *in vitro*. Even though the therapeutic plasma level of enoxaparin as anticoagulant clinically ranges between 1.3-2.7 μ M, the anticancer ability of enoxaparin in our study was detected at concentrations beyond clinical relevance, which is a limitation of our study. However, previous *in vitro* studies have reported that the minimum concentration of enoxaparin to inhibit proliferation and/or migration is 22 μ M (26, 28, 41), which is in the range of enoxaparin concentrations used in this study. The higher concentrations of enoxaparin necessary to obtain an antiproliferative and/or antimigratory effect *in vitro* may be related to either the addition of FBS in media (or other similar additives), or the fact that the hematological and immune systems are absent from *in vitro* systems. Therefore, further studies are necessary to determine if the therapeutic enoxaparin concentrations used for the treatment of VTE also confer an antiproliferative and/or antimigratory effect on cancer cells *in vivo*.

Many potential mechanisms that may explain the ability of LMWHs to interfere with tumor progression in various cancer types have been reported (7, 8, 42, 43). These proposed mechanisms fall under two major categories: i) coagulation-dependent and ii) coagulation-independent. The coagulation-dependent mechanisms by which LMWHs interfere with tumor development are related to their anticoagulant function through inhibition of thrombin generation. Thrombin converts fibrinogen to fibrin, and fibrin can lodge around tumor cells facilitating their survival, adhesion, and metastasis (44-46). Fibrin also provides a scaffold for angiogenesis (47). Therefore, attenuation of thrombin generation by LMWHs has the potential to reduce tumorigenesis. The coagulation-independent mechanisms are related to LMWHs' direct effect on tumor biology through alteration of PARs signaling, the fundamental link between blood coagulation and cancer pathogenesis (10, 27, 48). Since thrombin is a PAR-1

Figure 4. Effect of PAR-1 gene knockdown in the A549 cell line. BrdU ELISA assay was used to measure (A) cell proliferation of A549 cells with or without PAR-1 siRNA transfection for 72 h. Data are presented as a mean $\pm$ S.D. of four independent experiments. *** p <0.0001 versus control group or versus respective treated groups (ANOVA). (B) Inhibitory effect of enoxaparin (22, 44 and 66 μ M) on A549 cell proliferation after Silencing PAR-1 gene. Data are presented as a mean $\pm$ SD of three independent experiments. ** p =0.0001 and *** p <0.0001 versus non-siRNA treated control group (ANOVA). Absorbance was measured at 450 nm using the microplate reader SYNERGY/HT.

Figure 5. Effect of enoxaparin in activation of PAR-1 downstream signaling cascades. Western blot analysis was performed to examine the effect of 24-hour enoxaparin pretreatment (22, 44 and 66 μ M) on protein expression levels of p-ERK1/2: total ERK1/2 (A and B) and p-Akt: total Akt (C and D). In the data shown, A and C are graphical representations of three independent experiments, while B and D are blots of one representative experiment. All protein expressions were normalized to β -actin. Data presented as a mean $\pm$ SD of three independent experiments. * p <0.05 versus control group or # p <0.05 versus respective treated groups (ANOVA).

Figure 6. Effect of enoxaparin on MMP-2 level in A549 cell line. Effect of 48 h enoxaparin treatment on (A) MMP-2 mRNA expression levels measured by qPCR. All reactions were measured in duplicates and mRNA expressions were normalized to GAPDH mRNA. Data are presented as a mean $\pm$ S.D. of three independent experiments. ** p <0.0001 versus control group (ANOVA). B and C) MMP-2 protein expression levels measured by western blot. In the data shown, (B) is the graphical representation of four independent experiments, while (C) is a blot of one representative experiment. All protein expressions were normalized to β -actin. Data are presented as a mean $\pm$ S.D. of four independent experiments. * p <0.05 versus control group or # p <0.05 versus respective treated groups (ANOVA).

Figure 7. Inhibition of A549 cell proliferation by enoxaparin and doxorubicin. BrdU ELISA assay was performed after treatment of the A549 cell line with 100 nM doxorubicin or 44 μ M enoxaparin alone or in combination for 24 h. Absorbance was measured at 450 nm. Data are presented as a mean $\pm$ S.D. of three independent experiments. * p <0.0001 versus control group or versus respective treated groups (ANOVA).

activator, treatment with LMWHs could potentially mediate their antitumor effects by both mechanisms: i) inhibition of thrombin generation (coagulation-dependent) and ii) subsequently suppression of PARs activation and their mediated pathways (coagulation-independent). However, in our experimental conditions we found that thrombin protein levels in A549 were too low to consider the inhibition of thrombin as the mechanism responsible for enoxaparin antitumor effects (data not shown). Nonetheless, A549 cells express high levels of PAR-1. Collectively, our findings confirmed that treatment with enoxaparin reduces cell proliferation which is likely mediated independent of thrombin inhibition, but it depends on the PAR-1 receptor by interfering with two of its major downstream transduction pathways, the MAPK/ERK and PI3K/Akt signaling.

Beside its observed activity in reducing proliferation, we found that treatment with enoxaparin has an inhibitory effect on the migration of the A549 cells. We verified that enoxaparin-mediated down-regulation of the MAPK and PI3K, as evident by their decreased active downstream effectors, p-ERK1/2 and p-Akt, resulted in reduction of MMP-2 levels and inhibition of A549 cell migration. In support of our findings, it has been reported that MMP-2 is overexpressed in multiple highly invasive malignant cancers, including A549 lung cancer cells. MMP-2 has the ability to degrade collagen IV, which is a major component of ECM (34, 49, 50). Therefore, selectively inhibiting

Figure 8. Induction of apoptosis in lung adenocarcinoma A549 cells. Representative scatterplots (A) and graphical data (B) of the effect on apoptosis after 24 h exposure to: i) control, ii) 0.4% DMSO control, iii) 4 μ M staurosporine, iv) 100 nM doxorubicin (Dox), v) 44 μ M enoxaparin (Enox) and vi) combination of (Dox+Enox). Apoptosis studies were carried out using Annexin-V/PI double labeling and samples were processed by a flow cytometer. Data are presented as a mean $\pm$ SD of four independent experiments. * p <0.05 *** p <0.0001 versus control group or versus respective treated groups (ANOVA).

Figure 9. Cell-cycle analysis of A549 lung adenocarcinoma cell line. Representative histograms (A) and graphical data (B) of the effect on cell-cycle progression following 24-h exposure to: i) control, ii) 0.3% ethanol control, iii) 30 μ M genistein, iv) 100 nM doxorubicin (Dox), v) 44 μ M enoxaparin (Enox) and vi) combination of Dox+Enox. A549 cell distribution in the various cell-cycle phases was measured using PI staining as a marker for DNA content and the samples were run by a flow cytometer. Data are presented as a mean $\pm$ SD of three independent experiments. * $p < 0.05$ *** $p < 0.0001$ versus control group (ANOVA).

MMP-2 has been shown to reduce tumor migration and invasion (51-53).

Collectively, the *in vitro* and *in vivo* studies for the antineoplastic properties of enoxaparin and other LMWHs are promising. However, they are not precisely predictive for the

enhanced clinical outcomes among cancer patients, since these patients are also on a chemotherapeutic regimen before or after starting LMWH anticoagulant therapy. While most researchers have been focusing on studying the antitumoral effects of LMWHs alone, the number of studies examining the

Figure 10. Proposed mechanisms of enoxaparin's anticancer effects in the A549 cell line.

therapeutic benefits of combining a LMWH with anti-cancer agent is low. Nevertheless, there are ongoing clinical and preclinical studies concerned with the benefits of adding enoxaparin to a conventional chemotherapy in various malignancies, including lung cancer (54-58). However, to our knowledge, no study has been performed to determine the combined anticancer effect(s) of combination treatment with doxorubicin and enoxaparin in the A549 cell-line. Doxorubicin is a widely used chemotherapeutic agent in the treatment of lung cancer. However, it is highly associated with the development of drug resistance (59, 60) and a dose-dependent cardiotoxicity (61, 62). Therefore, to overcome doxorubicin's drug resistance, and reduce its toxicity profile, we tested the use of a low dose of doxorubicin with enoxaparin in A549 cells. Our data demonstrate that co-treatment with enoxaparin increases doxorubicin's inhibition of cell growth by enhancing doxorubicin-induced apoptosis, although enoxaparin alone does not alter apoptosis. On the contrary, doxorubicin-mediated cell cycle alteration is not influenced by enoxaparin. Other investigators have shown conflicting results with regards to enoxaparin's effect on apoptosis and cell cycle. In agreement with the data presented in the current study, high concentration of enoxaparin (88 μ M) did not affect apoptosis in human colon cancer HCT-116 cells but it induced G₀/G₁ phase arrest (63). Conversely, a different report has shown an increase in A549 cell apoptosis by enoxaparin at 44 μ M (41). Some factors that might influence the heterogeneity of the response to enoxaparin are: i) the experimental study design, ii) the type of cancer cells tested, cancer stage, iii) the presence of PARs receptors and thrombin expression, as well as iv) the concentration and duration of treatment. Additionally, Lean and his group have

found that enoxaparin actually consists of pro- and anti-proliferative oligosaccharides, and they hypothesized that even a small variation in the ratio of these saccharides can change the overall cellular response to enoxaparin (63). Unlike the sequence of their anticoagulant penta-saccharide, the exact sequence of enoxaparin (and other LMWHs) that is responsible for their antitumoral property is not yet identified. For this reason, different sources of commercially available LMWHs guarantee a predictable anticoagulant activity, but not the other potential anticancer effects. Therefore, more studies are necessary to identify the exact sequence responsible for the antitumoral activity.

In conclusion, our presented data add more evidence to the ability of enoxaparin to suppress lung cancer proliferation and migration, by down-regulating two major PAR-1 mediated pathways, the MAPK and PI3K. Clearly, enoxaparin does not have sufficient anti-cancer activity to be substituted for standard chemotherapy in cancer treatment. Nonetheless, the concept of adding enoxaparin to standard chemotherapeutic regimens might be a promising therapeutic approach, especially in the sub-population of lung cancer patients that have poor prognosis and/or are at a high risk of VTE. However, further studies are necessary to determine if the observed *in vitro* antiproliferative and antimigratory effects of enoxaparin are translatable in animal models of cancer.

Conflicts of Interest

The Authors declare that there are no known conflicts of interest associated with the work presented in this manuscript. Furthermore, the authors confirm that the funding provided for these studies did not influence the results in anyway.

Authors' Contributions

AA and MM conceived the idea for this project and planned the experiments. AA conducted all the experiments and drafted the manuscript under the mentorship and supervision of MM. All authors provided important scientific contributions to help shape this research, discussed the results, and reviewed the manuscript prior to submission.

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