Combinatorial Treatment of Tinzaparin and Chemotherapy can Induce a Significant Antitumor Effect in Pancreatic Cancer

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Research Article

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Abstract

Background: Pancreatic Cancer (PC) is recognized as a highly thrombogenic tumor, with over 20% of patients suffering from venous thromboembolism during the disease. Thus, low-molecular-weight heparin (LMWH) such as Tinzaparin is routinely used for PC patients.

On the basis of combinatorial therapy approach to treating highly malignant and refractory cancers such as PC, we hypothesized that Tinzaparin can augment the effectiveness of traditional chemotherapeutic drugs and induce efficient antitumor activity.

Methods: PANC-1 and MIAPaCa-2 were incubated alone or in combination with Tinzaparin, Nab-paclitaxel and gemcitabine. *In vivo* evaluation of these compounds was performed at NOD/SCID mouse using a model injected with PANC-1.

Results: Tinzaparin enhances the anti-tumor effect of Nab-paclitaxel and gemcitabine in mtKRAS PC cell lines via apoptosis at *in vitro* experiments. The triple combination acts through induction of apoptosis, reduction of the proliferative potential and angiogenesis hence contributing to a decrease in tumor volume observed *in vivo*. Tumor reduction observed in mice receiving the triple combinational chemotherapy, was 74.5% compared to the control. The triple regime provided an extra 8.2% and 24.3% tumor reduction compared to the control and the double combination (gemcitabine plus Nab-paclitaxel) respectively.

Conclusions: Combinatorial strategy can create novel therapeutic approaches for the treatment of patients with Pancreatic Cancer, achieving a better clinical outcome and prolonged survival. Further prospective randomized research is recommended and the investigation of various concentrations of Tinzaparin above of 150 UI/Kg, would potentially provide a valuable synergistic effect to the conventional therapeutic compounds.

Background

Pancreatic Cancer (PC) is the fourth leading cause of cancer death, with a five-year survival rate of 9% [1]. The two main types of cancer are adenocarcinoma (which accounts for about 85% of cases) and pancreatic endocrine tumors (which make up less than 5% of all cases). The incidence of pancreatic cancer has been steadily increasing in the recent years and it is predicted to be the second leading cause of death in Western countries by 2030 [2][3]. In spite of the extensive research that is performed, prognosis and therapeutic strategies have very slightly improved due to late diagnosis and limited responsiveness to potential compounds [4]. Causes of poor survival are considered to be the low effectiveness of adjuvant chemotherapy in surgery, the undetectable micro-metastases and the development of drug resistance [5]. These patients show a rapid progression of the disease and few of them survive more than a year. Even for patients with a diagnosed disease undergoing radical surgery, the median survival remains low at approximately a period of 18 months. Thus, despite the advances in

understanding the disease's basic biology, survival rates have remained substantially unchanged over the past 30 years [6].

Gemcitabine is currently the standard treatment option for advanced and metastatic PC and other types of cancers such as, small cell lung, bladder and breast. It was initially applied as an antiviral agent and later it was widely used as an anti-cancer chemotherapeutic agent for various solid tumors and lymphomas [7]. Despite its effectiveness as a drug to a significant number of cases, the different response rates, the increased toxicity and the development of resistance to some patients, remain important causes of ineffectiveness in recurrent tumors [8]. In order to compensate for these limitations, a high dose of gemcitabine (approximately 1000 mg / m²) is usually administered, but it causes severe side effects such as shortness of breath, neutropenia, nausea and kidney failure [9].

The combination of gemcitabine with Nab-paclitaxel has showed higher rates of response and increased survival in patients with pancreatic cancer. Nab-paclitaxel is a colloidal suspension of particles homogenized in human serum albumin-bound to paclitaxel. In mouse models, the combined treatment of gemcitabine and nab-paclitaxel resulted to increased concentration of gemcitabine in plasma and tumors [10]. Nab-paclitaxel is associated with the albumin receptor gp60 as well as with the secreted acid-rich and cysteine-rich SPARC protein, which is expressed in many cancer cells [11][12][13]. However, the combination of the two chemotherapeutic drugs, nab-paclitaxel and gemcitabine, has been shown to elevate hematological toxicity compared to monotherapy [14].

Tinzaparin, along with dalteparin and enoxaparin, belongs to the Low Molecular Weight Heparins (LMWH), which are derivatives of Unfractionated Heparins (UFH), through the depolymerization of heparin by heparinase enzyme [15]. They cannot inhibit thrombin, as they lack the specific pentasaccharide sequence necessary for interaction with antithrombin III, but they can attenuate coagulation factor Xa [16][17]. They offer a significant therapeutic advantage, as they have a more predictable dose-response to anticoagulant therapy, improved subcutaneous bioavailability, dose-dependent clearance, longer half-life, lower incidence of thrombocytopenia, and reduced requirement for laboratory monitoring [18].

Heparin administration has been extensively evaluated as a treatment for syndromes such as acute coronary heart disease, deep vein thrombosis (DVT) and pulmonary embolism (PE). It has also been studied for the prevention of venous thromboembolism (VTE) in several high-mobility patients [19]. In recent years, LMWH has successfully replaced UFH in both thromboprophylaxis and initial treatment of VTE. Studies on the efficacy and safety of Tinzaparin in DVT have concluded that it constitutes an effective therapeutic approach for the secondary prevention of thromboembolic events [7][20].

PC is recognized as a highly thrombogenic tumor, with over 20% of patients suffering from venous thromboembolism during the disease [21][22]. Chemotherapeutic agents are associated with thrombotic mechanisms, including the release of coagulants and cytokines from tumor cells, the production of toxic agents that act directly on the endothelium. Surgery, the first-line treatment for many cancer patients, is

known to activate the hemostatic system and in this way, cancer treatments activate coagulation cascade, promoting thrombosis and tumor growth [23].

Beyond their role in reducing VTE occurrence in pancreatic cancer patients, Tinzaparin may help to prolong survival by affecting tumor progression, metastasis formation, and angiogenesis based on *in vitro* studies but also *in vivo* studies on breast cancer [24][25]. This study aimed to determine the role of tinzaparin in pancreatic cancer. More specifically, we intended to decipher whether it contributes to the shrinkage of the tumor, especially after co-administration with gemcitabine and Nab-paclitaxel and identify the possible *in vitro* and *in vivo* mechanisms by which this inhibition is achieved.

Methods

Cell lines

Human pancreatic cancer cell lines, Panc-1 and MIA PaCa-2, were cultured according the guidelines of the American Type Culture Collection (ATCC). More specifically, cell lines were cultured in Dulbecco's Modified Eagle Medium (Life Technologies, USA) supplemented with 10% FBS, 100 U/mL Penicillin-Streptomycin and 2 mM L-glutamine.

Cell viability - XTT assay

The assessment of pancreatic cancer cell proliferation was performed with the XTT Cell Proliferation Assay Kit (10010200, Cayman Chemical, USA). Cells were seeded in a 96-well plate at a density of 103–105 cells/well in the aforementioned medium and cultured in a 100-μl medium with or without the tested drugs (1 μM gemcitabine, 1 μM Nab-paclitaxel, 2U/ml tinzaparin) in a CO₂ incubator at 37°C for 48h. Afterwards, 10 μl of XTT Mixture was added to each well and was mixed gently for 1 min on an orbital shaker. The plates were incubated for 2h at 37°C in a CO₂ incubator and the absorbance of each sample was measured using a microplate reader at 450 nm.

Western blotting

RIPA buffer was used for the preparation of whole cell lysates. The protein concentration was determined using the Bradford method (Bio-Rad, 5000006). A total of 25 μg of protein was resolved on SDS-PAGE and transferred to nitrocellulose membrane (Whatman, Scheicher & Schuell, Dassel, Germany). Membranes were incubated with primary antibodies overnight at 4°C. After incubation time, membranes were washed with TBS-T and incubated with the appropriate secondary antibody, for 1 h at RT. Antibodies were used against: VEGFR #9698, cleaved caspase-3 #9661 and PARP-1 #9542 (Cell Signaling) and Actin (sc-8035) from Santa Cruz. After incubation with HRP-conjugated secondary antibodies, the detection of the immunoreactive bands was performed with the Clarity Western ECL Substrate (Bio-Rad). Relative protein amounts were evaluated by a densitometry analysis using ImageJ software (La Jolla, CA, USA) and normalized to the corresponding actin levels.

In vivo experiments

All *in vivo* experiments were performed at NOD/SCID mice between 6 and 8 weeks of age. All procedures were carried out in accordance with the guidelines for animal experimentation following the European Union of the National and Kapodistrian University of Athens, Medical School Bioethics Committee in agreement with the European Union (approval no. 3233/26-06-2018). Panc-1 cell suspensions (1X10⁶) in 100 µL PBS were injected into the right flank of each mouse and allowed to grow for approximately 2 weeks to the point that they will be palpable. The mice were randomly divided into groups (n = 5 per group) for each treatment (control, tinzaparin, Nab-paclitaxel, Nab-paclitaxel + tinzaparin, gemcitabine, gemcitabine + tinzaparin, Nab-paclitaxel + gemcitabine, Nab-paclitaxel + gemcitabine + tinzaparin). We used tinzaparin at 10 mg/Kg, and it was administered daily, by subcutaneous injection (s.c.), Nab-paclitaxel at 25 mg/Kg and gemcitabine at 60 mg/Kg, twice per week by intraperitoneal injection (i.p.). The mice were euthanized, and tumors were measured and excised after 15 days of treatment. The tumor volume was calculated using the following formula: $1/2(\text{length} \times \text{width}^2)$.

Immunohistochemistry

Tumors from NOD-SCID mouse xenografts were fixed in 10% formalin solution and embedded in paraffin for sectioning at 4 µm. Sections were then deparaffinized in xylene, dehydrated through a graded ethanol series. Antigen retrieval was performed by heating the samples for 20 min at 95°C in citrate buffer (pH 6.0), and endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 min at room temperature (RT). Afterwards, the sections were washed with PBS and blocked with Normal Goat Serum 5% (NGS) for 1 hour. Then, sections were incubated with cleaved Caspase-3 antibody (rabbit monoclonal antibody, Cell Signaling, #9661), VEGFR-2 antibody (rabbit monoclonal antibody, Cell Signaling, #9698) at a dilution of 1:400 at 4°C overnight. After washing with PBS, the sections were incubated with biotinylated secondary antibodies (cat. no. 20775; Millipore) for 10 min at RT. Therefore, incubated with Streptavidin HRP (cat. no. 20774; Millipore) for 10 min at RT, and the reaction was visualized using 3,3'-diaminobenzidine. Eventually, the specimens were counterstained with Mayer's hematoxylin at RT for 1 min. Images were photographed with a Nikon Eclipse 80i microscope with a digital camera image system (Cellsens). Samples were blindly inspected by experienced pathologist.

Immunofluorescence

Initially, the sections were incubated with citrate buffer (pH 6.0) for antigen retrieval and then blocked in 5% NGS for 1 hour and incubated at a dilution of 1:400 at 4°C overnight with PCNA antibody (mouse monoclonal antibody, Cell Signaling, #2586). After washing with PBS, the sections were incubated with Alexa Fluor 568 secondary antibody (1:500, Molecular Probes). Sections were examined using an Olympus FV1000 confocal microscope.

Statistical analysis

Statistical significance was analyzed by the two-tailed Student t test. Values of $P < 0.05$ were considered to represent statistically significant group differences. All data represents mean $\pm$ SD. Microsoft Excel was used.

Results

Tinzaparin enhances the anti-tumor effect of Nab-paclitaxel and gemcitabine in mtKRAS PC cell lines

In our study, we examined the effect of co-treatment with Nab-paclitaxel (Nab-P), gemcitabine (G), Tinzaparin (T) in PC cell lines, PANC-1 and MIAPaCa-2. Both cell lines were exposed to 1 μ M Nab-P, 1 μ M G, 2UI/mL T alone or in combination for 48 hours and then, the cell viability was quantified by the XTT-viability assay. Cells without any drug administration were used as control. In PANC-1 cell line, Tinzaparin decreased cell viability around 20% whereas the combination of T + G and T + Nab-P decreased cell viability by 41% and 42.5% respectively. In the triple combinatorial scheme (Nab-P + T + G), the cell viability of PANC-1 was decreased by 55% after 48 hours.

In, MIAPaCa-2 cell line, Nab-P reduced cell viability by 21.5% and the combination of T + Nab-P and T + G lead to an additional reduction of 8.5%. The triple regime T + G + Nab-P resulted in a significant decrease of the total cell viability after 48 hours 40% compared to the untreated control. It appears that the triple combinatorial scheme of T + G + Nab-P, provides an extra reduction of cell viability in both cell lines than the agents alone (Fig. 1).

Decreased cell survival is the result of the stimulation in apoptotic mechanisms

In order to explore further the mechanisms by which the triple combination of T, G and Nab-P affects the viability of PC cell lines PANC1 and MIAPaCa-2, we performed Western Blot assays for specific apoptotic markers. More specifically the cells were exposed to 1 μ M Nab-P, 1 μ M G, 2 UI/mL T alone or in combination for 48 hours and cell lysates were quantified and used to detect two specific apoptotic markers, PARP-1 and caspase-3. Double combination of T + G or T + Nab-P slightly increased the cleaved caspase-3 and PARP in both cells lines. Triple combinatorial scheme of T + G + Nab-P in PANC1 and MIAPACA2 resulted in apoptotic cell death. The presence of apoptotic cell death was confirmed by the detection of PARP-1 cleavage and cleaved caspase-3 (Fig. 2). Our results highlight the vigorous antitumor activity of Tinzaparin when it is combined with Nab-paclitaxel and gemcitabine in *in vitro* experiments in PC models according to mutant KRAS profile.

In vivo experiments with xenograft NOD/SCID mouse model shows that Tinzaparin administration acts synergistically with chemotherapeutic drugs and provides a significant tumor reduction

Collectively of our *in vitro* experiments, we support the hypothesis that the combinatorial treatment with tinzaparin, gemcitambine and Nab-paclitaxel decreases the viability of PC cell lines. In light of these results, we tried to evaluate our *in vitro* results in xenograft mouse models. For xenograft studies, we used the Panc-1 PC cell line, bearing mutant KRAS, because it showed more reliable and significant results in

our *in vitro* experiments. Combinatorial treatment effectively decreased the tumor volume as shown in Fig. 3.

It is noteworthy that tumor reduction observed in mice receiving the triple combination chemotherapy, tumor reduction was 74.5% compared to the control. The inclusion of Tinzaparin to the triple regime compared to the double combination provided an additional 8.2% and 24.3% tumor reduction compared to the control and the double combination (gemcitabine plus Nab-paclitaxel) respectively. Similar tendency was observed after administration of Nab-paclitaxel plus Tinzaparin compared to control (extra 6.3% reduction) and when compared to Nab-paclitaxel alone (extra tumor reduction approximately 16.1%). Also, the combination of tinzaparin with gemcitabine led to a reduction of 11.8% compared to the control and 13.9% compared to gemcitabine alone (Fig. 3).

Following the tumor growth results, we sought to investigate the possible mechanisms responsible for the tumor growth attenuation of the triple combinatorial scheme on Panc-1 xenograft models. Immunohistochemistry studies showed that apoptotic cell death is triggered as it was measured by staining of the apoptotic marker cleaved caspase-3. Our results highlight the vigorous antitumor activity of Tinzaparin when it is combined with Nab-paclitaxel and Gemcitabine at *in vitro* and *in vivo* experiments in PC models according to mutant KRAS profile.

Immunofluorescence with PCNA shows that the triple combination has significant reduction in cell proliferation in mouse xenografts

Next, we performed immunofluorescence to the extracted tumors in order to examine the proliferation status of the cells with the use of PCNA marker. Tumors at untreated mice had as expected increased proliferation potential ($76.25\% \pm 5.68\%$ positive cells) but treated with the triple combination had 3.86-fold less proliferation capacity ($P < 0.0001$). When triple combination compared to Nab-P + G there was a reduction of proliferating cells by 30.01% ($P < 0.0001$) (Fig. 4A, B).

Stimulation of apoptotic pathways is one of the mechanisms leading to tumor reduction in vivo

The extracted tumors were further analyzed with immunohistochemistry with the use of an antibody against caspase-3. The percentage of apoptotic cells at the triple combination was $24.83\% \pm 3.63\%$ while at the control it was $2.75\% \pm 1.84\%$, a significant increase of 9.03-fold ($P < 0.0001$). Similar substantial increase of the apoptosis was observed (24.89% , $P = 0.0007$) when the triple combination is compared to the double, revealing the substantial synergistic effect of Tinzaparin (Fig. 5A, B).

Triple drug combination negatively affects tumor neoangiogenesis

Finally, an important factor for tumor growth is the formation of new vessels that would provide all the essential nutrients for its development. The effect of drug treatments was investigated by immunohistochemistry with the use of anti-VEGFR2. As pointed out at Fig. 6, there were a large number

of vessels formatted at the control sample but this phenomenon was reversed with the use of Tinzaparin, especially to the triple combination where less than 3 vessels were counted at each field of measurement.

Summarizing the above, we can conclude that the triple drug combination can lead to tumor growth reduction through pathways that affect cellular proliferation, apoptosis and neoangiogenesis. Despite the established role of the aforementioned drugs such as gemcitabine and Nab-paclitaxel in pancreatic cancer treatment, the administration of Tinzaparin acts synergistically to this effect and might provide novel therapeutic approaches.

Discussion

Pancreatic Cancer constitutes one of the most lethal diseases, with an average 5-year survival rate of less than 10% [1]. About 60–80% of patients are diagnosed with advanced cancer as it invades the surrounding tissues (locally advanced) or has disseminated outside the pancreas (metastatic) [26]. KRAS mutations comprise the most frequent alterations observed in pancreatic ductal adenocarcinoma (PDAC), therefore this type of cancer is frequency, is considered one of the most the most RAS-dependent of all cancers [27][28]. As the disease shows a very high mortality rate, it is imperative to discover novel and, more effective treatments, especially for those carrying the KRAS mutation.

The presence of desmoplasia is a feature of the pathogenesis and evolution of PC that represents 60–90% of the total tumor mass [29] and can be recognized mainly in both primary and metastatic areas of the tumor [30]. Fibroblasts are the predominant cell type within the TME, where they form a heterogeneous group of cells that contribute to ECM formation and support tumor growth [31]. Pancreatic stellate cells represent a subset of cancer-related fibroblasts present in a dormant state in healthy pancreatic tissue. Once activated, i.e., as a result of inflammation, injury, or tumor formation, they adopt a myofibroblast-type phenotype with high proliferative capacity and increased extracellular matrix protein secretion [32]. TME exerts various actions ranging from tumor formation, cancer spread and resistance to treatment [33]. Conventional drugs, such as gemcitabine, are not able to penetrate the rich and thick layer of TME and therefore lead to drug tolerance. TME is further characterized by severe hypoxia and, when combined with vascular compression induced by desmoplasia, triggers the process of angiogenesis to support the tumor's constant need for nutrients [34].

In our study, the PC line PANC-1 was selected, among other reasons, because this type of cells exhibit stromal characteristics and can provide a descent simulation of the real conditions of pancreatic cancer concerning the TME [35][36]. Taking into account that LMWHs can reduce fibrin formation and contribute to ECM degradation the result is the weakening of the TME. It could be hypothesized that when combined with drugs such as Nab-paclitaxel and gemcitabine that can penetrate the cancer cells more efficiently and eliminate them, there might be a synergistic effect with the action of LMWHs, something that is reflected in our *in vivo* results.

Tumors release vascular growth factors, such as VEGF and fibroblast basal growth factor (bFGF), which, along with other cytokines, stimulate angiogenesis by interacting with their high-affinity intracellular activity receptors. In humans, therapeutic doses of UFH actually cause increased levels of growth factors, such as plasma bFGF [18]. Heparin affects the activity of other factors involved in angiogenesis and tumor growth, in addition to VEGF and bFGF [37]. Growth factor TGF is a potent immunosuppressive and essential regulator of the growth, differentiation and adhesion of various cells. It is expressed in cancer cells and its overproduction is associated with an unfavorable prognosis.

LMWHs, on the other hand, can inhibit the binding of growth factors to their receptors while they are able to negatively affect the rate of angiogenesis [38]. The reduction of angiogenesis has been shown in an experimental model of human colon cancer, where tinzaparin administration 24 hours after angiogenesis stimulation by VEGF led to a reduction of the angiogenic index to the control level [39]. Tinzaparin exerts its anti-neoangiogenic activity as it appears to stimulate more production of Tissue Factor Pathway Inhibitor (TFPI) by epithelial cells than any other Low Molecular Heparin, inhibiting Tissue Factor (TF) and consequently VEGFR. However, LMWHs are known to inhibit heparanase, an endoglycosidase rarely expressed in normal tissue, while it is overexpressed in pancreatic tumors. The activity of LMWHs is demonstrated through the inhibition of VEGF-A and FGF-2 as well as the increased release of TFPI, suggesting an antagonistic role of LMWHs in the angiogenesis due to heparanase. Vascular endothelial growth factor A (VEGF-A) binding to the receptor tyrosine kinase VEGFR2 triggers multiple signal transduction pathways, which regulate endothelial cell responses that control vascular development [40].

There are several reports about the role of Tinzaparin in various types of cancer, where it appears to attenuate tumor growth but -most importantly-the metastatic process. Using a mouse pancreatic cancer cell line injected to mouse models, tinzaparin was able to inhibit tumor growth without affecting the thrombotic phenotype [41]. Another study demonstrated Tinzaparin's ability to upregulate the expression of E-cadherin in pancreatic tumor cells, a marker of decreased disseminating capacity and reduced metastatic potential [42]. Tinzaparin administration in mice injected with melanoma cells was capable of diminishing the metastatic cascade to distant organs by disturbing the P-, L-selectin and VLA-4/VCAM-1 interconnections. In addition, Tinzaparin suppressed the binding of malignant cells expressing the C-X-C chemokine receptor type 4- to their specific ligand on normal tissue, resulting in a substantial reduction in the dissemination of human breast cancer cells to the lung [43]. The antimetastatic properties of tinzaparin have also been observed in a B16 melanoma cell lung metastasis model in mice [15]. Administration of tinzaparin prior to the injection of melanoma cells resulted in lung tumor formation by 89% compared with controls whereas daily administration of tinzaparin for 2 consecutive weeks reached an additional reduction in lung tumor formation, reaching almost 96% [44].

Our results demonstrate, that tinzaparin reduces the expression of VEGFR2 resulting in fewer vessels formation in the tumor and, therefore, lower potential for nutrition. VEGFR2 activation stimulates cell proliferation and survival, activities that were inhibited in our experiments. We showed an important down-regulation of VEGFR2 and suppressed cell proliferation, observed with PCNA marker staining. Also,

our data revealed a reduction in the survival through the mechanism of apoptosis, as seen by the *in vitro* and *in vivo* experiments using caspase-3.

PaCT (Pancreatic Cancer & Tinzaparin) is a retrospective observational study that collects data regarding progression-free survival (PFS) in advanced or metastatic PC patients who received thromboprophylaxis with tinzaparin during chemotherapy with Nab-paclitaxel and gemcitabine. In this study, the median PFS was 7.9 months. Out of 14 similar studies (involving 2994 patients) identified via systematic search, it was settled that the weighted PFS of patients receiving Nab-P and G but no anticoagulation was 5.6 months. Therefore, patients receiving tinzaparin had 39.54% higher PFS than patients without thromboprophylaxis ($p < 0.05$). Tinzaparin was administered in a “hyper-prophylactic” of 10.000 Anti-Xa IU (175 UI/Kg) or even higher full treatment doses. Possible reasons for the increase in PFS were the treatment of venous thromboembolism, a prevalent condition in pancreatic cancer and the possible antitumor role of tinzaparin [45].

It should be noted here that the dose of tinzaparin administered (250UI / Kg) to the mice was the highest possible without bleeding, so it can be considered hyper-prophylactic as used in the PaCT study [41]. A clinical study using tinzaparin in patients with Non-Small Cell Lung Cancer did not have the expected PFS results, probably because the tinzaparin dose was 100UI / Kg, lower than the 175 UI / Kg used in the PaCT study [46].

Conclusions

Pancreatic cancer remains a considerable challenge in terms of its treatment. Delayed diagnoses, TME, tolerance to drugs such as gemcitabine are some of the causes of low survival of PC patients. Although life expectancy has increased in recent years with the administration of drug combinations, further improvement is required in order to find more effective treatments. Based on the results of the present study, we believe that the administration of tinzaparin in patients with pancreatic cancer who carry the KRAS mutation is a line of treatment following this direction. More specifically, co-administration with Nab-paclitaxel and gemcitabine in NOD / SCID mice experiments shows further tumor reduction through apoptosis, decrease of tumor vascularity and possibly weakening of desmoplasia resulting in greater penetration of the other chemotherapeutic drugs. Further prospective randomized research is recommended and the investigation of various concentrations of Tinzaparin above of 150 UI/Kg, would potentially provide a valuable synergistic effect to the conventional therapeutic compounds.

Abbreviations

DVT

deep vein thrombosis

i.p.

intraperitoneal

LMWH

low-molecular-weight heparin
PC
Pancreatic Cancer
PE
pulmonary embolism
PFS
progression-free survival
s.c.
subcutaneous
TF
Tissue Factor
TFPI
Tissue Factor Pathway Inhibitor
UFH
Unfractionated Heparins
VEGF
Vascular endothelial growth factor
VTE
venous thromboembolism

Declarations

Availability of data and material: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request

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Authors' contributions

All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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The manuscript has been read and approved by all named authors and there are no other persons who satisfied the criteria for authorship but are not listed.

The order of authors listed in the manuscript has been approved by all of us.

Ethics approval: All procedures were carried out in accordance with the guidelines for animal experimentation following the European Union of the National and Kapodistrian University of Athens, Medical School Bioethics Committee in agreement with the European Union (approval no. 3233/26-06-201

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Figures

Figure 1

Tinzaparin administration to drug combinations results in further decrease in cell viability Cell viability with XTT assay of the mutant KRAS pancreatic cancer cell lines PANC-1 and MIAPaCa-2 after 48 hours treatments with 1 μ M Nab-paclitaxel (Nab-P), 1 μ M gemcitabine (G) and 2 UI/mL Tinzaparin (T) alone or in combination. All data represents mean $\pm$ SD.

Figure 2

Tinzaparin administration to drug combinations leads to an additional increase of apoptosis. Western blot analysis of pancreatic cancer cell lines PANC-1 and MIAPaCa-2 after 48 hours treatments with 1 μ M Nab-paclitaxel (Nab-P), 1 μ M gemcitabine (G) and 2 UI/mL Tinzaparin (T) alone or in combination. Protein levels of apoptotic cell death were identified by antibody against PARP and cleaved Caspase-3. Protein levels were normalized against actin.

Figure 3

Tinzaparin, results in tumor growth inhibition in combination with chemotherapeutic drugs in pancreatic tumor xenografts (A) Mice were injected subcutaneously to the right flank with 0.1 mL PBS containing 106 PANC-1 human pancreatic cancer cells. After 15 days of treatment, mice were euthanized and tumors were collected for further analysis. (B) The diagram shows the tumor volume for each group (control, tinzaparin, Nab-paclitaxel, Nab-paclitaxel + tinzaparin, gemcitabine, gemcitabine + tinzaparin, Nab-

paclitaxel + gemcitabine, Nab-paclitaxel + gemcitabine + tinzaparin) during treatment. All data represents mean $\pm$ SD, (n = 5/group) (C) Representative tumors for each group with different treatment or without receiving treatment (control).

Figure 4

Tinzaparin, leads to reduced proliferation when used in combination with chemotherapeutic drugs (A) Immunofluorescence with PCNA antibody to 4 μ m paraffin sections. Representative images (PCNA-red spots and tissue-bright field) of each group (control, tinzaparin, Nab-paclitaxel, Nab-paclitaxel + tinzaparin, gemcitabine, gemcitabine + tinzaparin, Nab-paclitaxel + gemcitabine, Nab-paclitaxel + gemcitabine + tinzaparin) at 100X magnification, scale bar=100 μ m. (B) Diagram showing the percentage of positive stained cells. n=10/group. All data represent mean $\pm$ SD.

Figure 5

Tinzaparin, induces apoptosis when administered as part of conventional double or triple drug regimes (A) Immunohistochemistry with anti-caspase 3 antibody at 4 μ m paraffin sections. Representative photos of each group (control, tinzaparin, Nab-paclitaxel, Nab-paclitaxel + tinzaparin, gemcitabine, gemcitabine + tinzaparin, Nab-paclitaxel + gemcitabine, Nab-paclitaxel + gemcitabine + tinzaparin) 200X

magnification, scale bar=50 μ m. (B) The diagram shows the percentage of positive cells for caspase-3. n=10/group. All data represent mean $\pm$ SD.

Figure 6

Tinzaparin auguments further decrease in neoangiogenesis when added to double or triple combinations Representative photos of each group (control, tinzaparin, Nab-paclitaxel, Nab-paclitaxel + tinzaparin, gemcitambine, gemcitabine + tinzaparin, Nab-paclitaxel + gemcitambine, Nab-paclitaxel + gemcitambine + tinzaparin) after staining with anti - VEGFR2 at paraffin sections Symbols are denoted as: + low expression ++ mid expression +++ high expression ++++ abundant expression. n=5/group 200X magnification, scale bar=50 μ m.