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TITLE: "Enhancing the Efficacy of Chemotherapeutic Breast Cancer Treatment with Non-anticoagulant Heparins"

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not be construed as an official Department of the Army position, policy or decision unless so
designated by other documentation.
14. ABSTRACT  Studies with mice bearing MCF7-WT xenografts demonstrate that encapsulation of Dox, whether in targeted or non-targeted PLGA nanoparticles, improved anti-tumor efficacy in comparison to un-encapsulated Dox. In animals bearing MCF7-R (Dox-resistant) tumors, administration of Dox encapsulated in av 3-targeted nanoparticles or of Dox with non-anticoagulant heparin (NACH) are potent strategies for overcoming Dox resistance in animals bearing these aggressive human breast tumors. HPLC analyses of tumors and tissues from animals bearing MCF7-R tumors clearly demonstrate that LMWH or NACH increase the uptake of Dox into tumors but not other tissues at 3 and 24 hrs, at least double the amount observed with Dox alone. 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 chemotherapeutic uptake. In vitro studies to investigate possible mechanisms associated with LMWH improvement of Dox anti-tumor activity focused on cell migration, proliferation and viability. LMWH compounds did not substantially affect these parameters in vitro. It is likely that increasing chemotherapeutic uptake in vivo as demonstrated in HPLC studies represents one important mechanism of improved anti-tumor efficacy associated with co-administration of LMWH and Dox.

15. SUBJECT TERMS  Breast cancer; nano-particle-site-directed therapy; low molecular weight heparins (LMWH); non-anticoagulant heparins
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SUMMARY
Thrombotic complications are the second most common cause of mortality in cancer patients and fibrin deposition in the tumor microenvironment might play a key role in tumor progression and inference with tumor chemotherapeutic uptake. Treatments that target these processes may result in improved uptake of chemotherapeutic agents and subsequent inhibition of tumor growth and metastasis. Tissue Factor (TF) is frequently associated with aggressive behavior and poor outcome in tumors. We have previously demonstrated potent anti-tumor efficacy for various mechanisms that interfere with TF/VIIa. The purpose of this study was to investigate the effects of low molecular weight heparins (LMWH) and sulfated non-anticoagulant LMWH (S-NACH) on tumor chemotherapeutic uptake. Studies: (1) Nude mice xenograft A549 human lung carcinoma: LMWH or S-NACH at 10 mg/kg S.C. daily effectively limited tumor growth. (2) LCC6 human lung tumor xenograft model: Paclitaxel alone or in combination with Tinzaparin or S-NACH on tumor re-growth after discontinuation of treatment: Paclitaxel + S-NACH treatment showed significant (P<0.01) tumor growth suppression and improved survival when compared to Paclitaxel. (3) Biodistribution studies: animals were injected with LMWH S.C. daily for 5 days (10 mg/kg) then injected i.v. with [3H]-Paclitaxel. LMWH increased [3H]-Paclitaxel uptake into LCC6 tumors with tumor: muscle ratios several fold greater than that of [3H]-Paclitaxel alone at 24 hrs post injection. 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. (4) HPLC studies of tumor uptake of Doxorubicin (DOX in mice treated with 10 mg/kg of LMWH or S-NACH for 10 days followed by Doxorubicin (2.5 mg/kg). Both LMWH and S-NACH significantly (P<0.01) increased the uptake of chemotherapeutic agent DOX in MCF7 DOX resistant tumors by 1.5-2 folds but not in heart or lung tissues, confirming the findings obtained with another agent [14C]-Paclitaxel. Conclusions: LMWH or S-NACH increased chemotherapeutics uptake and hence chemoresponse. Protocols utilizing adjuvant or neo-adjuvant therapy with LMWH or S-NACH could lead to increase tumor chemo responsiveness and overcoming tumor chemo resistance.

INTRODUCTION:
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). There is substantial literature support for the use of low molecular weight heparin (LMWH) for treating coagulation disorders in cancer patients. However, recent prospective clinical trials have demonstrated that they provide significant advantages in terms of progression-free and overall survival in certain cancers and in certain subgroups of patients (4-8). Data from in vitro and experimental animal models also provide encouraging scientific rationales for application of these agents to control tumor growth and metastasis (9-12). Survival advantages have not been seen in breast cancer trials, perhaps because increased bleeding times in these patients constitute a dose-limiting side effect. We have developed novel non-anticoagulant heparin (NACH) compounds that have minimal effects on hemostasis (13). In the Specific Aim 1 of studies proposed, we will test the ability of NACH to improve the efficacy of chemotherapy treatment without affecting hemostasis in a mouse 01thotopic model of breast cancer using Doxorubicin-sensitive or -resistant MCF7 human breast cancer cells. In some studies, we will use PEG-PLGA nano-particles for targeted drug delivery of NACH and Doxorubicin (Dox), directing therapeutic treatments to the tumor neovasculature by attaching av3 antibody to the surface of nanG-particles. In Specific Aim 2, we will study the possible mechanisms of action of NACH with respect to tumor growth, invasiveness, and chemotherapeutic drug sensitivity in vitro using the same drug-sensitive and drug-resistant MCF7 breast cancer cell lines that were used for Specific Aim 1. New nano-particle technology provides unprecedented opportunities for addressing areas in breast cancer research due to the utilization of biodegradable/biocompatible polymeric materials for carrying therapeutic agents to tumor sites. Nano-therapy studies have just begun in man and
experimental studies such as the one proposed here will provide support for application of such regimens for the treatment of breast cancer in the future and advance research in this field.

RESEARCH ACCOMPLISHMENTS DURING YEAR 2

STATEMENT OF WORK:
The research studies to be performed are summarized in two Specific Aims: **Specific Aim: In vivo** studies will be performed for proof-of-concept that NACH will significantly increase the efficacy of breast cancer treatment with Doxorubicin. Female athymic mice will have either drug-resistant or drug-sensitive MCF7 human breast cancer cells implanted orthotopically into the fourth mammary gland. Treatment modalities will be evaluated for their effects on tumor growth, metastasis and tumor-associated angiogenesis, and will include nano-particle targeted vs. untargeted therapies as outlined in Research Strategy. Bleeding times will be performed in a cohort of animals to confirm that NACH treatments have minimal effects on hemostasis in tumor-bearing animals. **Specific Aim 2:** We will study the possible mechanisms of action of NACH with respect to tumor growth, invasiveness, and chemotherapeutic drug sensitivity *in vitro* using the same drug-sensitive and drug-resistant MCF7 breast cancer cell lines that were used for Specific Aim 1. Treatments will be tested to evaluate their effects on TFPI-1/-2 and sirt1 expression by Western blotting and real-time RT-PCR. We will evaluate the functional consequences of these treatments by studying tumor growth (cell number) and invasiveness (migration), and determine whether increased levels of TFPI-1/-2 or reduced levels of sirt1, if they are associated with these treatments, result in increased drug-sensitivity in the treated cell lines.

**Brief summary of work accomplished in Year 2**
The first series of studies were done with non-nanoparticle-encapsulated treatments. Studies were performed to test the antitumor efficacy of LMWH and NACH with and without Doxorubicin (Dox) in animals bearing **MCF7-WT tumors.** To quantify tumor growth data, we evaluated the time in Days to form tumors 2000 mm^3^ in size. Dox treatment resulted in a statistically significant increase in the time for tumors to reach 2000 mm^3^ and resulted in increased survival of the animals in comparison to untreated control groups. Both Dox + ENOX and Dox + NACH groups significantly attenuated tumor growth to 2000 mm^3^, even though the differences between these groups and Dox alone did not reach statistical significance. In addition, animal survival in these groups was improved relative to animals receiving Dox alone. The increased survival ratios and lengthening of the time required for tumor growth indicate that these treatments may have the potential for increasing the efficacy chemotherapeutic agents. Studies were also performed in mice bearing **MCF7-R (Dox-resistant) tumors.** Both Dox and Dox + ENOX treatments significantly increased the time interval to the development of tumors sized 2000 mm^3^, while Dox + NACH group shows less effective protection with a P value approaching but not reaching statistical significance. This observation was corroborated by comparison of the number of surviving animals in Dox and Dox + ENOX categories. Bleeding times were determined by standard methodology to evaluate whether treatment with LMWHs would increase this indicator of disrupted hemostasis. Although there was a trend toward increased bleeding time in Dox + ENOX groups, for both MCF7 and MCF7-R groups, there were no statistically significant differences between the ENOX groups and the other groups. However, approximately half of the animals in ENOX groups had bruising at the sites of injection.

**RESULTS OF STUDIES PERFORMED DURING YEAR 2**
The results presented in this report were obtained during the second year of funding. We have obtained a no-cost extension (until May 2010) which will be utilized to continue the studies detailed below, repeating experiments as necessary to obtain statistical significance. The studies to be performed during the no-cost extension are within the scope of the approved Statement of Work.
A. IN VIVO STUDIES

Treatment Groups

1. Controls: no treatment
2. Doxorubicin (Dox) alone
3. Control nanoparticle: without surface targeting and containing no therapeutic treatment
4. av 3-targeted nanoparticle + Dox
5. av 3-targeted nanoparticle + Dox + Enoxaparin
6. av 3-targeted nanoparticle + Dox + NACH

General Experimental Design

- Tumor cell lines MCF7- wild type or MCF7-R were injected into 4th mammary fat pad of nude mice.
- Animals were randomized into treatment groups after tumor implant when tumors were palpable or at least 80 mm3 in size. Treatments were begun.
- Treatments: Dox 2.5 mg/kg SC injection on alternate days; Enoxaparin or NACH 10 mg/week daily; For combination therapy: 2.5 mg/kg Dox + either 10 mg/kg Tinzaparin OR NACH.
- In experiments with nanoparticle formulations, see Fig 2, all treatments were administered on alternate days for the course of the experiment.
- Tumor measurements were obtained at 1-2 day intervals, starting after tumor implantation.
- Animals were sacrificed tumor weights obtained.
- Tumors and lungs were fixed for histology and immunohistochemistry to evaluate tumor-associated angiogenesis.

RESULTS

Fig 1: Anti-tumor efficacy of Nanoparticle formulations encapsulating Dox with and without LMWHs vs. un-encapsulated Dox in mice with MCF7-WT tumors. Mice were inoculated with 1.4 x 10^7 MCF7-WT cells. Treatments were begun 10 days after tumor cell inoculation as shown in legend. Tumor volume measurements were obtained over time course shown. Values are Mean tumor volume in mm3 ± SEM, n = 8 mice/group. In Control (untreated) group and in animals treated with void nanoparticles, tumors continued to increase in volume. As expected, un-encapsulated Dox (black triangles) effectively inhibited this Dox-sensitive tumor. However, Nano-Dox (white triangles) or av 3-targeted Nano-Dox (black squares) treatments showed similar patterns of inhibition and appeared to be more effective in inhibiting tumor growth than un-encapsulated Dox (p <0.05) over the tumor growth period encompassing 5-15 days. Targeted Nano-Dox particles containing either LMWH (black diamonds) or NACH (white squares) showed similar levels and patterns of inhibition to that of Nano-Dox treatments. With respect to hemostasis, nanoparticles that contained LMWH Enoxaparin, but not NACH, caused bruising at the site of injection. Conclusions: Encapsulation of Dox, whether in targeted or non-targeted nanoparticles improved anti-tumor efficacy in comparison to un-encapsulated Dox. In this initial experiment, all nanoformulations showed similar patterns of inhibition with no significant statistical differences from each other in the nanoformulations treatment groups. Future studies will investigate long-term effects of these treatments on survival and tumor growth and.
will evaluate whether there are differences in the efficacies of LMWH- or NACH-containing nanoparticles.

**Fig 2:** Anti-tumor efficacy of Nanoparticle formulations encapsulating Dox with and without LMWHs vs. un-encapsulated Dox in mice with MCF7-R Tumors. Mice (8/group) were inoculated with 3 x 10^6 MCF7-R cells. Treatments, as shown in legend, were begun after tumors had reached a size of 50-100 mm^3. Tumor volumes were measured daily. Animals were euthanized when Controls reached a size of 2000 mm^3 (as per IACUC approval).

**Panel A** illustrates the effects of treatments on tumor volume over time. Dox-treated animals showed the same pattern of tumor growth as untreated controls, as expected with this Dox-resistant tumor. Encapsulation of Dox in a αvβ3-targeted nanoparticle (closed triangle) significantly improved the anti-tumor efficacy of Dox in these Dox-resistant tumors. Further, substantial inhibition was observed with NACH (open triangles), Dox + NACH (closed squares), and Dox + LMWH (closed diamonds) groups, even without encapsulation in nanoparticles, suggesting that LMWH or NACH can improve the anti-tumor activity of Dox, even in a drug-resistant tumor. One possible mechanism that could be involved in this effect is discussed below in studies of LMWH and chemotherapeutic uptake (Fig 10). The most effective anti-tumor agent was αvβ3-targeted Dox + NACH nanoparticle treatment that was responsible for slowing tumor growth rate and limiting tumor size. **Panel B** illustrates tumor weights measured after animals were euthanized. All treatment groups were superior to Dox treatment alone (p < 0.001), and the pattern of inhibition paralleled that observed with the tumor growth curves in Panel A. Treatment group 6, αvβ3-targeted Dox + NACH showed inhibition that was significantly different from all treatment groups, p value vs. other groups was at least <0.02. **Conclusions:** These studies demonstrate that encapsulating Dox in αvβ3-targeted nanoparticles or administering it with NACH represent potent strategies for overcoming Dox-resistance in animals bearing aggressive chemoresistant human breast tumor. In future studies we will repeat and expand these studies to optimize dosing regimens and drug concentrations.

**Fig 3:** Toxicity of treatment groups: Effects of Nanoparticle and non-nanoparticle formulations on weights of animals bearing MCF7-R Tumors. The weights of animals receiving various treatments were monitored as evidence of toxicity of the specific treatments. Major toxic effects are defined as those associated with loss of 20% of body weight over the period of treatment. As shown in Fig 3, there were no significant changes in animal body weight with any treatment over the time course of the study. Although injections of formulations containing LMWH compound Enoxaparin was associated with bruising at the injection site, this was not the
case with formulations containing NACH, whether encapsulated in nanoparticles or not. At the doses of LMWH compounds used there was no evidence of bleeding within the organs or body cavity. **Conclusions:** Treatments with LMWH compounds with or without Dox are well-tolerated by mice bearing this aggressive human breast tumor over the time course of treatment. Additional evaluations will be performed including histologic examinations of tumors to evaluate tumor angiogenesis and organs for metastasis. Activated partial thromboplastin time (aPTT) and anti-Xa testing (frozen plasma samples) will be performed for all animals to evaluate effects on hemostasis.

**B. IN VITRO STUDIES**

These studies were performed to investigate the possible mechanisms involved in inhibition of tumor growth by LMWH compounds with and without Dox. The same cell lines, MCF7-WT and MCF7-R, used for the in vivo studies were used for all in vitro studies. The assays were designed to evaluate individual aspects of the processes involved in tumor growth and metastasis, namely migration and viability/proliferation.

**Fig 4: Effects of Dox with and without NACH on migration of MCF7-WT cells.** To determine whether the agents used in the in vivo studies had any direct effects on the ability of cancer cells to migrate, we utilized a 96-well Neuroprobe™ migration assay. The bottom chamber contained either 10% fetal calf serum (FCS) as chemo-attractant stimulus or no stimulus for negative control (NBS). MCF7-WT or MCF7-R cells were plated on the surface of a filter unit suspended above the chemoattractant and allowed to attach for 10 minutes. Test agents were added to the upper chamber with the cancer cells and migration was quantified 5 hrs later. **Total** migration was defined at maximum migration occurring in the absence of inhibitors. Values are expressed as % migration relative to positive control (10% FCS) ± SEM.

Migration of MCF7-WT cells in the absence of chemoattractant (NBS) is minimal (1-2%) while 10% FCS stimulates maximum migration. XT199, a small-molecule inhibitor of integrin (av 3)-dependent migration, effectively inhibits cancer cell migration (87%). Dox alone (red bars) inhibits migration in the range of 25-45% and was statistically significant, p vs. Total < 0.01. NACH (1 ug/ml group (green bars): NACH at 1 ug/ml alone inhibits migration by 34% but combinations ofNACH with Dox are not additive and no more effective than either agent alone. NACH group, (10 ug/ml (yellow bars): NACH stimulates migration as effectively as 10% FBS and combinations with 0.1 uM and 0.5 uM Dox are only modestly inhibitory. **Conclusions:** While both Dox and NACH cause moderate inhibition of cancer cell
migration, they are not as effective as XT199 which acts through integrin-dependent mechanisms to limit migration. While these agents show some inhibition of migration, it is unlikely that they exert their effects primarily through processes affected by cellular migration.

Fig 5: Effects of Dox with and without NACH on migration of MCF7-R cells. Evaluation of migration was performed as described above for MCF7-WT cells.

Migration of MCF7-R cells in the absence of chemoattractant (NBS) is minimal (1-2%) while 10% FCS stimulates maximum migration. XT199, a small-molecule inhibitor of integrin (CXV 3)-dependent migration, effectively inhibits cancer cell migration (95%). Dox alone (red bars) significantly inhibited migration of MCF7-R cells, with inhibition ranging from 29-59%, p vs. Total < 0.001. NACH (1 ug/ml group (green bars): NACH at 1 ug/ml alone inhibits migration by 32% but combinations of NACH with Dox are not additive and no more effective than either agent alone. NACH, 10 ug/ml group (yellow bars); NACH showed modest inhibitory activity (11-25%) and combinations with Dox did not improve inhibitory properties. Conclusions: Although Dox alone shows significant inhibition of MCF7-R cell migration, addition of NACH did not improve this effect, and in some cases appears to blunt the inhibition that might be expected from corresponding doses of Dox.

Fig 5: Effects of NACH on migration of human endothelial cells Human dermal microvessel endothelial cells (HDMEC) or Human umbilical vein endothelial cells (HUVEC). Because tumor angiogenesis is a critical component for tumor growth and metastasis, we investigated whether NACH had a direct effect on 2 endothelial cell lines using a standard method for evaluating endothelial cell migration – migration across a scratched monolayer.
EC growing as a confluent monolayer were treated for 2 days with NACH at concentrations from 1-100 ugjml. Control monolayers were untreated. A scratch was made across the EC monolayer, the media was changed and cells were re-treated with NACH. Images were captured on the day of injury and each day afterward. Number of cells migrating across the scratched area was quantified and expressed as Average number of Cells/ mm² ± SEM, n = 3. Although it appeared that certain concentrations of NACH decreased migration of EC across an injured monolayer, the data were not statistically significant. 

**Conclusions:** From the above result of this assay NACH does not appear to inhibit the migratory properties of endothelial cell line, microvessel or large vessel EC. Because there is considerable variability in the data, these studies will be repeated and a confirmatory Neuroprobe migration assay will be performed to determine whether LMWH compounds affect the ability of EC to migrate, and thus participate in a crucial aspect of the process of angiogenesis.

**Fig 6: Effect of NACH and Dox on Proliferation of MCF7-WT cells.** MCF7-WT cells were seeded in culture plates and cultured in the absence (Control) or the presence of varying concentrations of NACH alone, Dox alone, or combinations of both drugs for 2 days. Cells were trypsinized and counted. Data are expressed as Cell Number ± SD. Control (blue bar); NACH alone at concentrations from 0.1-50 ugjml (green bars); Dox alone (red bars); Dox 0.1 uM + varying concentrations of NACH (yellow bars); Dox 0.5 uM + varying concentrations of NACH (purple bars). See below.

Dox alone effectively inhibits proliferation of these Dox-sensitive cells Dox 0.1 uM (58%); Dox 0.5 uM (72%). NACH alone is not inhibitory with maximum inhibition of 18% seen in 5 ugjml group. In 0.1 uM Dox combination group all NACH doses are less effective than the corresponding dose of Dox alone. Likewise, in the 0.5 uM Dox combination group, the combination of Dox with 1 ugjml of NACH provides a similar degree of inhibition as Dox alone (66%) but the effect is likely due to the Dox component of the combination. **Conclusions:** NACH does not augment the inhibitory activity of Dox on the proliferation of MCF-WT cells.
Fig 7: Effect of NACH and Dox on Proliferation of MCF7-R cells. MCF7-R cells were seeded and cultured in the absence (Control) or the presence of varying concentrations of NACH alone, Dox alone, or combinations of both drugs for 2 days. Experimental protocol and legend is the same as for Fig. 6 above.

Dox alone inhibited proliferation at either concentration. NACH alone, at the highest concentration 80 ng/ml stimulated cell proliferation. Lower concentrations caused no significant inhibition. In the 0.1 µM Dox + NACH groups (yellow bars) proliferation inhibition to equivalent to or less than Dox alone. This was true for the 0.5 µM Dox + NACH group as well. Conclusions: NACH alone showed modest inhibition of cancer cell proliferation that did not reach statistical significance. Dox combinations with NACH did not result in improved inhibition of MCF7-R proliferation. Rather, % inhibition was likely due to the Dox component of the combination.

Fig 8: Effects of combinations of Dox with LMWH or NACH on MCF7-R viability 2 days after treatment. MCF7 cells were seeded then allowed to attach before treatments as indicated below in legend. Viability was determined using standard MTT assay and expressed % Viability (relative to untreated controls) ± SEM.
Dox at 0.1 uM did not affect viability, and cell number increased over the 2-day period in culture. A ten fold concentration, 1 uM was required to affect cell viability. NACH alone (green bars) at all concentrations stimulated cell growth relative to untreated controls. NACH over a range of concentrations combined with 0.1 uM Dox (yellow bars) did not affect viability; However NACH combined with 1 uM Dox (lt. yellow bars) effectively killed cells; this effect was likely due to Dox itself. Likewise, LMWH alone (dark blue bars) did not affect cell viability but appeared to stimulate cell growth. When combined with 0.1 uM Dox (lt. blue bars), only the highest dose of LMWH, 100 ug/ml, affected cancer cell viability; combinations with 1 uM Dox (aqua bars) resulted in significant inhibition, likely due to the Dox in the combination. Conclusions: LMWH or NACH given in a range of concentrations from 1-100 ug/ml have no effects on viability of MCF7-R cells. In combination with Dox, the LMWH compounds show only viability effects that are attributable to the concentration of Dox utilized in the combination.

Future Directions and on-going studies: From the in vitro studies performed to date, there are no clear indications that LMWH compounds function through modification of migratory properties of cancer cells, inhibit cell growth or affect viability. Cell samples from all of these studies have been or will be processed for Western blotting and RT-PCR analysis to determine whether treatments are associated with changes in TFPh/2, sirt1, or sirt7 protein or gene expression. When all the data have been analyzed, results will be presented at meeting, in papers and as part of the final report for this grant.

Possible mechanism for LMWH anti-tumor efficacy: Although the in vitro studies shown in this report have not demonstrated a likely mechanism for the effects of LMWH on tumor growth in vivo, we have performed HPLC evaluations of Dox concentrations in tumor and organs from mice bearing MCF7-R tumors. We believe that the results shown below provide an insight into the increased efficacy of chemotherapeutics treatments that include LMWH.

Fig 10: HPLC Determination of Dox in Tissue and tumors of mice bearing MCF7-R Xenografts and treated with LMWH or NACH.

To determine whether LMWH compounds increase the uptake of chemotherapeutic agents into tumors, mice were pre-treated with 10 mg/kg of LMWH or NACH for 5 days followed by DOX (2.5 mg/kg). Three or 24 hrs later animals were euthanized and tissues obtained for HPLC determination of DOX. Calibration curves were generated from DOX spiked into blank tumor tissue and extracted with solvent (Methanol: Chloroform, 1:4).
**Conclusions:** Both LMWH and NACH significantly increased the uptake of chemotherapeutic agent DOX in MCF7 Dox-resistant tumors by 1.5-2 fold but not in heart or lung tissues (* p<0.01). These findings confirm data previously obtained by us with another chemotherapeutic agent [124-I]-Paclitaxel in an aggressive human lung tumor LCC6. In that study there was a constant positive enhancement effect between controls and heparin groups, with at least a two-fold increase in tumor to muscle ratio. 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.

**KEY RESEARCH ACCOMPLISHMENTS:**
- In vivo experiments that are part of Specific Aim 1 have continued and now include the nanoparticle formulation studies.
- Most of the in vitro experiments for Specific Aim 2 have been performed and include evaluations of treatment group effects on cancer cell and endothelial cell migration, cancer cell proliferation, and viability.
- Cell samples from these in vitro experiments have been or will be processed for Western blotting and RT-PCR to evaluate effects on protein and mRNA for key molecules TFPI-1 and -2, and sirtl and sirt7. All data will be compiled and presented in the final report in May 2010.
- Tumor and tissue samples from in vivo experiments have been or will be processed for histology to evaluate tumor angiogenesis.

**REPORTABLE OUTCOMES:**
- An abstract was presented as a poster at the Era of Hope Meetings in June 2008. The data in this report and additional studies performed as an outgrowth of the concepts supported in this grant will be presented at additional meetings in the future.
- Data from these studies, when complete, will be submitted for publication.
• Collaborative studies are underway with a group at Roswell Park to pursue the therapeutic potential of LMWH in cancer, specifically their effects on uptake of chemotherapeutic agents. These studies, supported by a Phase I SBIR grant, have potential for submission for a Phase II grant.

CONCLUSIONS:
**In vivo studies** of mice bearing MCF7-WT xenografts demonstrate that encapsulation of Dox, whether in targeted or non-targeted nanoparticles improved anti-tumor efficacy in comparison to un-encapsulated. Studies performed with animals bearing MCF7-R tumors demonstrate that encapsulating Dox in av 3-targeted nanoparticles or administering it with NACH represent potent strategies for overcoming Dox-resistance in animals bearing aggressive chemo-resistant human breast tumor. In future studies we will repeat and expand these studies to optimize dosing regimens and drug concentrations. HPLC analyses of tumors and tissues from animals bearing MCF7-R tumors clearly demonstrate that the LMWH and NACH increase the uptake of Dox into tumors but not other tissues, at least double the amount observed with Dox alone at 3 and 24 hrs. 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.

**In vitro studies** to investigate possible mechanisms associated with LMWH improvement of Dox anti-tumor activity focused on cell migration, proliferation and viability. LMWH compounds did not substantially affect these parameters *in vitro*. It is likely that increasing chemotherapeutic uptake *in vivo* as demonstrated in HPLC studies represents one important mechanism of improved anti-tumor efficacy associated with co-administration of LMWH and Dox.

REFERENCES

APPENDICES:

- List of employees
- Abstract presented at the Era of Hope Meeting in June 2008
- Abstracts (2) presented at the ASCO meeting 2009
- First year progress report
- Poster presented at ASCO 2009

Employees:

Abdelhadi Rebbaa
Ahmad Aljada
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Site Directed Delivery of Chemotherapy and Non Anticoagulant Sulfated Heparin in Breast cancer
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There is substantial literature support for the use of low molecular weight heparins (LMWH) for treating coagulation disorders in cancer patients. Recent prospective and retrospective clinical trials have also demonstrated that they provide significant advantages in terms of progression free and overall survival in certain cancers and in certain subgroups of patients. LMWH treatments are often associated with increased bleeding, constituting a dose limiting effect. We have developed novel non anticoagulant heparin (NACH) compounds that have minimal effects on hemostasis (El-Naggar and Mousa, US Patents 6,908,907 B2, (2005), and 10, 667,216, (2003). We have evaluated them for efficacy vs. tumor growth and metastasis and have begun to investigate the mechanisms involved in anti-tumor activities. Modified sulfated LMWH with weak or no anticoagulant activities were still highly effective in inhibiting angiogenesis and metastasis, demonstrating that anticoagulation is not essential for attenuation of angiogenesis or metastasis. The modified heparins were characterized with respect to their ability to release endothelial tissue factor pathway inhibitor (TFPI) and inhibit selectin-mediated interactions, molecular components that have been shown to modulate tumor growth, tumor angiogenesis and metastasis. One of these modified heparin compounds that showed significant activity in a selectin mediated tumor cell adhesion assay was also highly effective in reducing tumor burden in mice with MC-38 colon carcinoma and D16-BL6 melanoma (>70%) and in reducing the number of metastatic foci (>65%) in these animals. We also investigated the efficacy of NACH compounds on growth factor-induced angiogenesis in a mouse Matrigel model in which new vessel growth was quantified by measuring hemoglobin concentration extracted from the Matrigel plug. Values are Means ± SEM. Matrigel alone: 0.57±0.12; bFGF + Matrigel: 7.27 ± 1.18; NAC-S-8: 0.86 ± 0.10. This sulfated compound which demonstrated no anticoagulant activity in aPTTI and TEG assays, reduced capillary formation to baseline levels. These data demonstrate that non-anticoagulant heparin compounds exhibit a profile of anti tumor activities without disrupting normal hemostasis. Site-directed therapy with non-anticoagulant heparins (NACH) and chemotherapy would allow for optimization of treatments in the tumor microenvironment. In studies that are currently underway, we are targeting the sites of tumor neovascularization using a biodegradable nanopatticulate system made up of a blend of MPEG-PLGA (methoxy-polyethylene glycol poly(lactide-co glycolide) and maleimide-PEG PLGA. These nanopaticles have their surfaces conjugated to alpha.v beta3 antibody and contain chemotherapeutic agent Doxorubicin, with or without NACH. Preliminary data indicate that repeated administration of sulfated non-anticoagulant heparin compound at 1.0 mg/kg S.C. daily for up to 14 days in conjunction with doxorubicin caused no bruising at the injection site, whereas Enoxaparin showed injection site bruising in >80% of the mice. The use of NACH agents that are co-encapsulated with chemotherapeutic agents could minimize the toxic side effects of the chemotherapy while delivering a combination of effective therapeutic agents directly to the tumor.
ABSTRACT


Background: In comparison to low molecular weight heparin (LMWH), non-anticoagulant heparin (NACH), originally developed in our laboratory, has minimal effects on hemostasis. Encapsulation of chemotherapeutic agents and NACH in biodegradable nanoparticles has tremendous potential to increase the survival rate among the breast cancer patients. Furthermore, custom-made nanoparticles with a targeted moiety on the surface will enable us to increase the efficacy and decrease the adverse toxicity of doxorubicin.

Method: PLGA-PEG nanoparticles co-encapsulating NACH and doxorubicin were synthesized by double emulsion solvent evaporation method. The in vitro efficacy of these nanoparticles was examined in MCF-7 doxorubicin resistant (MCF-7R) cells by cell viability (MTT) assay. Confocal microscopy was used to examine the uptake of αvβ3 antibody conjugated nanoparticles in human dermal microvascular endothelial cells (HDMEC), which are known to over express αvβ3 integrins.

Results: Size measurement by DLS revealed these nanoparticles co-encapsulating doxorubicin and heparins to be > 300 nm. Data from the MTT assays in MCF-7R cells are shown Table 1. In vivo data using in mice xenograft (MCF-7R) are shown in Table 2 (Amounts of doxorubicin and NACH injected in all cases were 0.625 mg/kg and 2.5 mg/kg body weight).

Table 1

<table>
<thead>
<tr>
<th>Formulation</th>
<th>% Cell Viability</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>2.0</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>95.8</td>
<td>2.4</td>
</tr>
<tr>
<td>NACH</td>
<td>132.2</td>
<td>4.6</td>
</tr>
<tr>
<td>Doxorubicin-NACH</td>
<td>111.0</td>
<td>2.9</td>
</tr>
<tr>
<td>Nano-Doxorubicin</td>
<td>110.0</td>
<td>2.8</td>
</tr>
<tr>
<td>Nano-NACH Nano-</td>
<td>158.5</td>
<td>4.2</td>
</tr>
<tr>
<td>Doxorubicin - NACH</td>
<td>66.5*</td>
<td>9.5</td>
</tr>
</tbody>
</table>

Table 2:

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Tumor Weight (g)</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.5</td>
<td>0.37</td>
</tr>
<tr>
<td>Void nanoparticles</td>
<td>1.9</td>
<td>0.49</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>1.3</td>
<td>0.15</td>
</tr>
<tr>
<td>Nano-Doxorubicin</td>
<td>1.2</td>
<td>0.10</td>
</tr>
<tr>
<td>Nano-Doxorubicin-&lt;Xv3</td>
<td>0.8*</td>
<td>0.12</td>
</tr>
<tr>
<td>Nano-Doxorubicin-NACH-av 3</td>
<td>0.5*</td>
<td>0.22</td>
</tr>
</tbody>
</table>

Conclusion: PLGA-PEG nanoformulations co-encapsulating NACH and doxorubicin exhibit superior in vitro efficacy compared to non-encapsulated drugs in the MCF-7R cell line. Confocal imaging in HDMEC cells indicates that these nanoparticles have the potential to be used for site specific delivery to the tumor neovascularization these findings were later supported by in vivo data. Significant decrease in tumor weight was observed in the mice (MCF-7R), when treated with <Xv3 conjugated nanoparticles co-encapsulating doxorubicin and NACH compares to its non encapsulated counterpart.
Effects of novel heparin-derived compounds on tumor uptake of chemotherapeutics and chemoresponse


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1The Pharmaceutical research Institute, Albany College of Pharmacy and Health Sciences, Albany, NY; 2 Dept. Nuclear Medicine, SUNY Buffalo; 3Roswell Park, Buffalo NY

Thrombotic complications are the second most common cause of mortality in cancer patients and fibrin deposition in the tumor microenvironment might play a key role in tumor progression and inference with tumor chemotherapeutic uptake. Treatments that target these processes may result in improved uptake of chemotherapeutic agents and subsequent inhibition of tumor growth and metastasis. Tissue Factor (TF) is frequently associated with aggressive behavior and poor outcome in tumors. We have previously demonstrated potent anti-tumor efficacy for various mechanisms that interfere with TF/VIIa. The purpose of this study was to investigate the effects of low molecular weight heparins (LMWH) and sulfated non-anticoagulant LMWH (S-NACH) on tumor chemotherapeutic uptake. Studies: (1) Nude mice xenograft A549 human lung carcinoma: LMWH or S-NACH at 10 mg/kg S.C. daily effectively limited tumor growth. (2) LCC6 human lung tumor xenograft model: Paclitaxel alone or in combination with Tinzaparin or S-NACH on tumor re-growth after discontinuation of treatment: Paclitaxel + S-NACH treatment showed significant (P<0.01) tumor growth suppression and improved survival when compared to Paclitaxel. (3) Biodistribution studies: animals were injected with LMWH S.C. daily for 5 days (10 mg/kg) then injected i.v. with [124-I]-Paclitaxel. LMWH increased [124-I]-Paclitaxel uptake into LCC6 tumors with tumor: muscle ratios several fold greater than that of [124-I]-Paclitaxel alone at 24 hrs post injection. 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. (4) HPLC studies of tumor uptake of Doxorubicin (DOX in mice treated with 10 mg/kg of LMWH or S-NACH for 10 days followed by Doxorubicin (2.5 mg/kg). Both LMWH and S-NACH significantly (P<0.01) increased the uptake of chemotherapeutic agent DOX in MCF7 DOX resistant tumors by 1.5-2 folds but not in heart or lung tissues, confirming the findings obtained with another agent [124-I]-Paclitaxel. Conclusions: LMWH or S-NACH increased chemotherapeutics uptake and hence chemoresponse. Protocols utilizing adjuvant or neo-adjuvant therapy with LMWH or S-NACH could lead to increase tumor chemo responsiveness and overcoming tumor chemo resistance.
Year 1 Progress

INTRODUCTION:
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). There is substantial literature support for the use of low molecular weight heparin (LMWH) for treating coagulation disorders in cancer patients. However, recent prospective clinical trials have demonstrated that they provide significant advantages in terms of progression-free and overall survival in certain cancers and in certain subgroups of patients (4-8). Data from in vitro and experimental animal models also provide encouraging scientific rationales for application of these agents to control tumor growth and metastasis (9-12). Survival advantages have not been seen in breast cancer trials, perhaps because increased bleeding times in these patients constitute a dose-limiting side effect. We have developed novel non-anticoagulant heparin (NACH) compounds that have minimal effects on hemostasis (13). In the studies proposed, we will test the ability of NACH to improve the efficacy of chemotherapy treatment without affecting hemostasis. In some studies, we will use PEG-PLGA nano-particles for targeted drug delivery of NACH and Doxorubicin (DOX), directing therapeutic treatments to the tumor neovascularature by attaching alpha v beta 3 antibody to the surface of nano-particles. New nano-particle technology provides unprecedented opportunities for addressing areas in breast cancer research due to the utilization of biodegradable/biocompatible polymeric materials for carrying therapeutic agents to tumor sites. Nano-therapy studies have just begun in man and experimental studies such as the one proposed here will provide support for application of such regimens for the treatment of breast cancer in the future and advance research in this field. These studies will be performed in a mouse orthotopic model of breast cancer using Doxorubicin-sensitive or -resistant MCF7 human breast cancer cells.

BODY: RESEARCH ACCOMPLISHMENTS DURING YEAR 1

Statement of work
The research studies to be performed during the first year of this grant are summarized in Specific Aim 1: Female athymic mice will have either drug-resistant or drug-sensitive MCF7 human breast cancer cells implanted orthotopically into the fourth mammary gland. Treatment modalities will be evaluated for their effects on tumor growth, metastasis and tumor-associated angiogenesis, and will include nano-particle targeted vs. un-targeted therapies as outlined below. Bleeding times will be performed in a cohort of animals to confirm that NACH treatments have minimal effects on hemostasis in these tumor-bearing animals. Evaluations will include determinations of the size of tumors, quantification of metastases and tumor-associated angiogenesis. For evaluation of metastases, lungs will be removed from the thoracic cage en bloc and lung seeding will be assessed by counting macroscopically the number of pulmonary tumor nodules on the entire surface of the lungs, and microscopically by histology of lung sections. Bleeding times will be performed using standard methodology. Tumor-associated angiogenesis will be evaluated using endothelial cell-specific CD31jPECAM staining.

Treatment Groups
7. Controls: no treatment
8. Doxorubicin (DOX) alone
   g. DOX + Enoxaparin (ENOX)
10. DOX + LMWH compound NACH
11. Control nanoparticle: without surface targeting and containing no therapeutic treatment
12. Targeted nanoparticle + DOX
13. Targeted nanoparticle + DOX + Enoxaparin
14. Targeted nanoparticle + DOX+ NACH

Experimental Design
• Tumor cell lines MCF7 – wild type or MCF7-R (DOX-resistant) were injected into 4th mammary fat pad of nude mice.
• Animals were randomized into treatment groups 7 days after tumor implant when tumors were palpable or so mm^3 in size. Treatments were begun.
• Treatments: DOX 2.5 mg/kg SC injection 3xjweek (Monday, Wednesday, Friday); Enoxaparin or NACH 10 mg/kg sfweek (Monday – Friday); For combination therapy: 2.5 mg/kg DOX + either 10 mg/kg Tinzaparin OR NACH.
• Bleeding times were determined 3 hrs post initial dose of either Enoxaparin or NACH. Each animal was subjected to bleeding time testing only once in the course of the experiment.
• Tumor measurements were obtained at 3-4 day intervals, starting Day 8 after tumor implantation.
• Animals were sacrificed tumor weights obtained.
• Tumors and lungs were fixed for histology and immunohistochemistry to evaluate tumor-associated angiogenesis.
• Body cavities were examined for the presence of metastases and observations recorded.

Results
To date, only the first half of the study has been performed – non-nanoparticle targeted treatments. Nanoparticles have been prepared and will be utilized for the second half of the study.

Figure 1: Tumor volume plots MCF7 tumors. Plots are shown for individual animals implanted with MCF7 (wild type) tumor cells and treated as designated on each plot and described above. Tumor measurements were begun at 8 days post-implant and continued every 3-4 days until animals were euthanized.
Data show that DOX-treated animals showed improved survival but here was variability in animal responses in both this treatment group and in DOX + ENOX groups. However, animals in the DOX + NACH group showed a more unified responses, with clusters of animals showing similar tumor growth rates. To quantify these data, we evaluated the time in Days to form tumors 2000 mm3 in size. Comparison of treatments for anti-tumor efficacy is shown below in Table 1 below. Data are also summarized with respect to number of animals surviving at 2 separate time points, Day 25 and Day29.

Table 1 Anti-tumor efficacy of LMWH treatment in combination with DOX in nude mice with MCF7 tumors

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Days to 2000 mm3 Mean±SEM (range)</th>
<th>Pvalueb (Control)</th>
<th>Pvaluec (DOX)</th>
<th>Surviving animals Day25</th>
<th>Surviving animals Day29</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20.57± 1.17 (17-25)</td>
<td>-</td>
<td>0.04</td>
<td>3/8</td>
<td>0/8</td>
</tr>
<tr>
<td>DOX</td>
<td>24-75± 1.46 (21-31)</td>
<td>0.04</td>
<td>-</td>
<td>5/9</td>
<td>3/9</td>
</tr>
<tr>
<td>DOX+ ENOX</td>
<td>25.11±1.18 (21-29)</td>
<td>0.016</td>
<td>0.850</td>
<td>7/10</td>
<td>5/10</td>
</tr>
<tr>
<td>DOX+ NACH</td>
<td>26.33 ±1.03 (22-32)</td>
<td>0.003</td>
<td>0.39</td>
<td>8/10</td>
<td>5/10</td>
</tr>
</tbody>
</table>

a Treatment of nude mice beginning day 8 after implant of MCF7 tumor cells
b based on comparison of each group vs. control using two-tailed t-test
c based on comparison of each group vs. DOX using two-tailed t-test

Data in Table 1 demonstrate that DOX treatment resulted in a statistically significant increase in the time for tumors to reach 2000 mm3 and resulted in increased survival of the animals in comparison to untreated control groups. Both DOX + ENOX and DOX + NACH groups significantly attenuated tumor growth to 2000 mm3, even though the differences between these groups and DOX did not reach statistical significance. In addition, animal survival in these groups was improved relative to animals receiving DOX alone. The increased survival ratios and lengthening of the time required for tumor growth indicate that these treatments may have the potential for increasing the efficacy chemotherapeutic agents and should be pursued. These studies will be repeated to confirm the findings and increase the likelihood of attaining statistical significance in comparison to DOX alone treatments.
**Figure 2: Tumor volume plots MCF-R tumors.** Plots are shown for individual animals implanted with MCF7-R doxorubicin-resistant tumor cell line and treated as designated on each plot and described above. Tumor measurements were begun at 8 days post-implant and continued every 3-4 days until animals were euthanized.

Data show that all three treatments provided some survival advantage in comparison to untreated animals. DOX treatment alone prolonged survival and in some animals may have decreased the rate of tumor growth, although considerable variation in response was observed in this group. In DOX+ ENOX group, the response to treatment appeared to be more consistent than in the DOX group. In the DOX + NACH group only 3/10 animals survived at Day 25 and 2/10 at Day 29 suggesting that this treatment did not improve the efficacy of DOX alone. In this group data were clustered in the time points encompassing 8-22 Days. However, two animals in this treatment group showed decreased tumor growth rate and increased survival. The survival data for these animals (Table 2 below) in the DOX + ENOX and DOX + NACH groups is different from that observed in Table 1 for animals bearing the MCF7 tumors.

To quantify these data, we evaluated the time in Days to form tumors 2000 mm3 in size. Comparison of treatments for anti-tumor efficacy is shown below in Table 2 below. Data are also summarized with respect to number of animals surviving at 2 separate time points, Day 25 and Day 29.
Table 2: Anti-tumor efficacy of LMWH treatment in combination with DOX in nude mice with MCF7-R tumors

<table>
<thead>
<tr>
<th>Treatment group a</th>
<th>Days to 2000 mm3Mean±SEM (range)</th>
<th>Pvalueb (Control)</th>
<th>Pvaluec (DOX)</th>
<th>Surviving animals Day25</th>
<th>Surviving animals Day29</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20.1 ± 0.81 (16-23)</td>
<td>-</td>
<td>0.050</td>
<td>2/10</td>
<td>0/10</td>
</tr>
<tr>
<td>DOX</td>
<td>24.56 ± 1.85 (15-32)</td>
<td>0.050</td>
<td>-</td>
<td>7/10</td>
<td>4/10</td>
</tr>
<tr>
<td>DOX+ENOX</td>
<td>24.37 ± 1.25 (21-29)</td>
<td>0.014</td>
<td>0.936</td>
<td>6/10</td>
<td>3/10</td>
</tr>
<tr>
<td>DOX + NACH</td>
<td>22.5 ± 1.18 (18-29)</td>
<td>0.11</td>
<td>0.366</td>
<td>3/10</td>
<td>2/10</td>
</tr>
</tbody>
</table>

a Treatment of nude mice beginning day 8 after implant of MCF7-R tumor cells
b based on comparison of each group vs. control using two-tailed t-test
c based on comparison of each group vs. DOX using two-tailed t-test

Data demonstrate that both DOX and DOX + ENOX treatments significantly increase the time interval to the development of tumors sized 2000 mm3, while DOX + NACH group shows less effective protection with a P value approaching but not reaching statistical significance. This observation is corroborated by comparison of the number of surviving animals in DOX and DOX + ENOX categories.

These studies will be repeated for confirmation, and because of the variability of responses, repeat studies may allow us to determine whether there are statistically significant differences among treatment groups.

Table 3 Bleeding time studies performed on all treatment groups. Bleeding times were determined by standard methodology (14) to evaluate whether treatment with LMWH would increase this indicator of disrupted hemostasis. Because each animal was subjected to bleeding time testing only once in the course of the experiment, each treatment group consisted of sub-groups A and B (5 animals per sub-group), total n per treatment group = 10. Table 3 summarizes the bleeding time data expressed as minutes and seconds ± SD, Although there was a trend toward increased bleeding time in DOX + ENOX groups, for both MCF7 and MCF7-R groups, there were no statistically significant differences between the ENOX groups and the other groups. However, approximately half of the animals in ENOX...
groups had bruising at the sites of injection.

**KEY RESEARCH ACCOMPLISHMENTS:** (bulleted list)

- Experiments for the first part of Specific Aim 1 (non-nanoparticle agents) have been performed. Data are presented in this report.
- Bleeding tests for these studies have been performed. Data are presented in this report.
- Tumor and lung tissue has been obtained from all animals and is being processed for examination. Data will be presented when complete.
- Nanoparticle formulations for the second part of Specific Aim 1 have been prepared and characterized. They are ready for use in targeted-nanoparticle agent studies.

**REPORTABLE OUTCOMES:**

- An abstract has been accepted for presentation as a poster at the Era of Hope Meetings in June 2008. The data in this report and additional studies performed as an outgrowth of the concepts supported in this grant will be presented.
- Data from this study, when complete, will be submitted for publication.
- Collaborative studies are underway with a group at Roswell Park to pursue the therapeutic potential of LMWH in cancer, specifically their effects on uptake of chemotherapeutic agents. These studies, supported by a Phase I SBIR grant, have potential for submission for a Phase II grant.

**CONCLUSIONS:** LMWH compounds given together with chemotherapeutic agent doxorubicin decreased tumor growth rate and prolong survival in animals bearing MCF7 wild-type tumors. These agents appear to be less effective in animals bearing doxorubicin-resistant tumors. Bleeding times determined on animals in all treatment groups did not show statistically significant differences. However, animals in ENOX groups showed increased bruising at the sites of injection. These studies will be repeated, and studies with alpha v beta 3-targeted nanoparticle formulations will be performed to compare the efficacies of non-targeted and nanoparticle-targeted therapies.

**APPENDICES:**

The abstract to be presented at the Era of Hope Meeting in June 2008 is included in the Appendix.

**REFERENCES**


EFFECTS OF NOVEL HEPARIN-DERIVED COMPOUNDS ON TUMOR UPTAKE OF CHEMOTHERAPEUTICS AND CHEMORYMPONSE

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1 The Pharmaceutical Research Institute, Albany College of Pharmacy and Health Sciences, Albany, NY; 2 Vascular Vision Pharmaceutical Co., Rensselaer, NY; 3 Dept. Nuclear Medicine, SUNY Buffalo; 4 Roswell Park, Buffalo, NY

INTRODUCTION

Thrombotic complications are the second most common cause of mortality in cancer patients, and fibrin deposition in the tumor microenvironment could play a key role in tumor progression and influence with chemotherapeutics uptake. Treatments that target these processes may result in improved uptake of chemotherapeutic agents and subsequent inhibition of tumor growth and metastasis.

Low molecular weight heparins (LMWHs) are used for treating coagulation disorders in cancer patients. These treatments are often associated with increased bleeding, constituting a dose-limiting effect. Availability of Non-anticoagulant heparins (NACH) would allow for optimization of these treatments in the tumor microenvironment, and could increase the efficacy of the chemotherapeutic response. We have developed novel NACH compounds that have minimal effects on hemostasis (Eil-Hegger, MM and Mousa, SA: US Patent 6,908,907 B2, June 21, 2005, and Mousa SA, US Patent 10, 667,216, Sept 19, 2003).

Increased Tissue Factor (TF) expression is frequently associated with aggressive behavior and poor outcome in tumors. LMWHs exert intravascular anticoagulant and anti-thrombotic effects through the release of tissue factor pathway inhibitor (TFPI), with limited effects on hemostasis.

The purpose of this study was to investigate the effects of LMWH and sulfated non-anticoagulant C-MPINOH on tumor uptake chemotherapeutics and chemomycympose.

TEST AGENTS AND METHODS

LMWH tinzaparin or oxidized sulfated LMWHs (S-NACH) or SS-NACH) were tested with or without Paclitaxel (PACL) or Doxorubicin (DOX) in mouse tumor xenograft models. Test agents were also evaluated for effects on chemotherapeutic uptake into tumors and tissue using two independent methods: Gamma counting of radioisotopically labeled Pacl and HPLC quantification of DOX. Methods and Experimental Designs are provided for individual figures.

RESULTS

CONCLUSIONS:

Both LMWHs effectively limited the growth of this tumor. These results were obtained in the absence of standard chemotherapeutic agents, and provided support for the concept that this anti-tumor effect could potentially augment the effectiveness of chemotherapeutic agents, perhaps allowing for lowering the doses required for these toxic agents.

LMWH tinzaparin or oxidized sulfated LMWHs (S-NACH) or SS-NACH) were tested with or without Paclitaxel (PACL) or Doxorubicin (DOX) in mouse tumor xenograft models. Test agents were also evaluated for effects on chemotherapeutic uptake into tumors and tissue using two independent methods: Gamma counting of radioisotopically labeled PACL and HPLC quantification of DOX. Methods and Experimental Designs are provided for individual figures.

FIG 1: LMWH AND NACH INHIBIT TUMOR GROWTH IN MOUSE XENOGRAFT MODEL OF LUNG CANCER. HUMAN BREAST TUMOR XENOGRAFTS. Nude mice (nu/nu) were injected with LCC human breast carcinoma cells (R C. daily at 10 mg/kg) over the course of the experiment.

CONCLUSIONS: Both LMWHs effectively limited the growth of this tumor. These results were obtained in the absence of standard chemotherapeutic agents, and provided support for the concept that this anti-tumor effect could potentially augment the effectiveness of chemotherapeutic agents, perhaps allowing for lowering the doses required for these toxic agents.

CONCLUSIONS: Highest levels of (124) I-Paclitaxel accumulation at 24 hrs were in liver, gut and stomach. All 3 groups treated with LMWH tinzaparin, S-NACH or SS-NACH showed greater % Injected dose (% ID) in tumors than in Controls. See Table 2 above for tumor to muscle ratios.

CONCLUSIONS: Highest levels of (124) I-Paclitaxel accumulation at 24 hrs were in liver, gut and stomach. All 3 groups treated with LMWH tinzaparin, S-NACH or SS-NACH showed greater % Injected dose (% ID) in tumors than in Controls. See Table 2 above for tumor to muscle ratios.

CONCLUSIONS: Both LMWHs and S-NACH significantly (P<0.01) increased the uptake of chemotherapeutic agents DOX in MCF7. DOX resistant tumors at 1-2 fold but not in heart or liver tissues, confirming the findings obtained with another chemotherapeutic (117) Paclitaxel. LMWH compounds increased DOX concentration at both 3 and 24 hrs in tumors but not in other tissues.

CONCLUSIONS:

LMWHs and S-NACH showed potent anti-tumor efficacy, and treatments were associated with slowing of tumor growth and prolonged survival after discontinuation of PACL treatments.

• LMWHs significantly increased chemotherapeutics uptake into tumors but not other tissues.

• Protocols utilizing adjuvant or neo-adjuvant therapy with LMWH or S-NACH could lead to increased tumor chemoresponsiveness, overcoming tumor resistance to chemotherapeutic agents.

TABLE 1: ANTITUMOR EFFICACY OF LMWH COMPOUNDS ALONE AND IN COMBINATION WITH PACL IN NUDE MICE WITH LCC HUMAN BREAST TUMOR XENOGRAFTS. Nude mice (nu/nu) were injected with LCC human breast carcinoma cells (R C. daily at 10 mg/kg) over the course of the experiment. Measurements were taken weekly for tumor size using two independent methods: Gamma counting of radioisotopically labeled Pacl and HPLC quantification of DOX. Methods and Experimental Designs are provided for individual figures.

TABLE 2: TUMOR TO MUSCLE RATIOS FOR UPTAKE OF (124)I-PACL.

TUMOR TO MUSCLE RATIO (AVG)

CONCLUSIONS:

Both LMWHs and S-NACH significantly (P<0.01) increased the uptake of chemotherapeutic agents DOX in MCF7. DOX resistant tumors at 1-2 fold but not in heart or liver tissues, confirming the findings obtained with another chemotherapeutic (117) Paclitaxel. LMWH compounds increased DOX concentration at both 3 and 24 hrs in tumors but not in other tissues.