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Low-Anticoagulant Heparins in the Treatment of Metastasis

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1. Introduction

Metastasis is a spread of cancer cells to a distant location from the primary tumor. This process involves a complex series of events similar to those that occur during inflammation. The events of metastasis can be divided into four major steps. First, cancer cells proliferate and transform to acquire motility and ability to invade basement membrane to reach blood vessels. Second, cancer cells penetrate blood vessels due to increased vascular permeability facilitated by inflammatory mediators and enter the circulation. Third, in the circulation cancer cells are shielded by host cells (platelets and neutrophils) to escape surveillance by immune cells and survive shear forces of the bloodstream. Next, integrin-mediated arrest of circulating cancer cells occurs on the endothelial surface before extravasation. Once cancer cells extravasate, heparanase from cancer cells degrades heparan sulfates of the extra cellular matrix. The cleavage of heparan sulfates release growth factors that stimulate cancer cell growth as well as angiogenesis.

The Receptor for Advanced Glycation Endproducts (RAGE) belongs to the immunoglobulin superfamily of cell surface molecules. The receptor's name, RAGE, was coined for its ability to bind its first described ligand, advanced glycation end products (AGEs), which accumulate in physiological (aging) and pathological disorders such as diabetes (Schmidt et al., 1992). RAGE is a pattern recognition receptor for its ligation to structurally unrelated ligands that include Mac-1, HMGB1 and S100 /calgranulins. Similar to immunoglobulin, RAGE contains an extracellular structure with a V-type binding region and two C-type regions. Immediately following the C-type region is a transmembrane region and a short cytoplasmic domain (Fig. 1). The important roles played by RAGE in inflammation, diabetes, Alzheimer's disease and cancer have been discussed in detail (Ellerman et al., 2007; Logsdon et al., 2007; Schmidt et al., 2001; Sims et al., 2010; Tang et al., 2010). RAGE is ubiquitously expressed in tissues and inflammatory cells at low levels in homeostasis and its expression is increased in stress conditions. RAGE expression is observed in many tumors, including brain, breast, colon, lung, prostate, pancreatic, ovarian cancers, lymphoma and melanoma (Hsieh et al., 2003; Logsdon et al., 2007) and elevated levels of RAGE have been reported in colon (Sasahira et al., 2005), prostate (Ishiguro et al., 2005) and gastric cancers

(Kuniyasu et al., 2002). In contrast, RAGE levels are down-regulated in lung cancer relative to levels in the normal lung tissue. These observations have led to studies to delineate the pathophysiologic involvement of RAGE in cancer. Notably, AGEs are involved in development and progression of cancers (Abe et al., 2007; Sebekova et al., 2007).

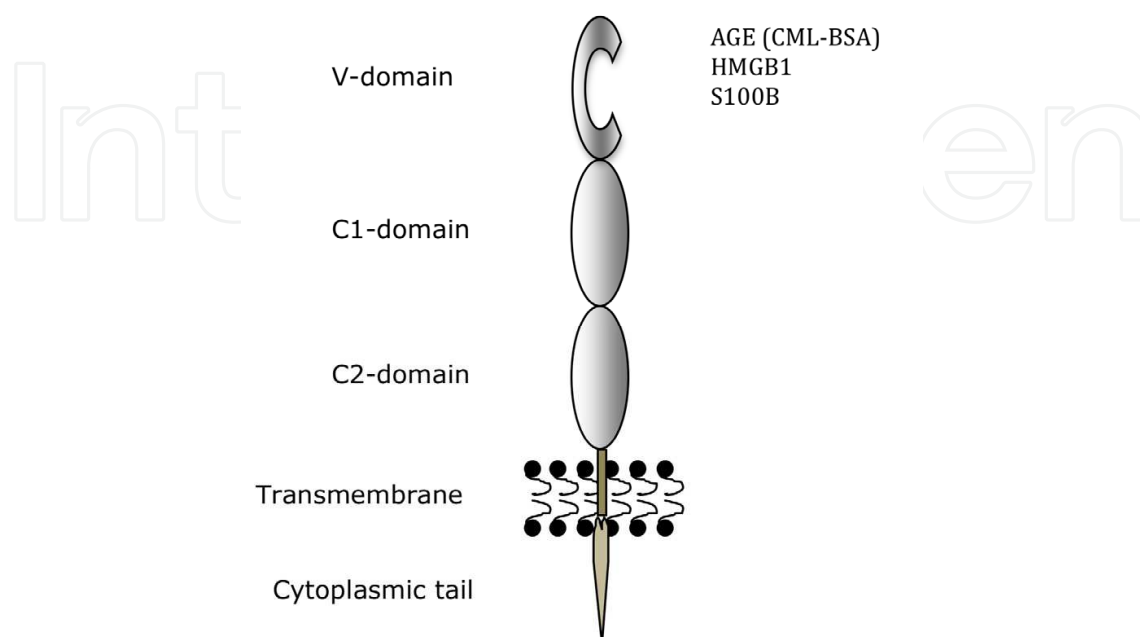


Fig. 1. RAGE domains and ligands involved in cancer. RAGE has ligand-binding V-domain followed by two C-domains of similar to immunoglobulins, a transmembrane region and cytoplasmic tail.

Heparin was discovered in 1916 (McLean, 1916)), and has been used almost exclusively as an anticoagulant in medicine. However, shortly after its initial discovery, it was reported that heparin had an inhibitory effect on tumor growth in animals (Goerner, 1930). This initial observation led to intensive investigation with regard to heparin's anticancer and anti-metastatic properties (Casu et al., 2010; Casu et al., 2008; Hettiarachchi et al., 1999; Kragh et al., 2005; Kragh & Loechel, 2005; Lapierre et al., 1996; Lever & Page, 2002; Ono et al., 2002; Ornstein & Zacharski, 1999; Smorenburg et al., 1996; Stevenson et al., 2005; Stevenson et al., 2007; Vlodavsky et al., 2006; Yip et al., 2006; Yoshitomi et al., 2004). The usefulness of heparin as an anticancer drug has been hindered by its anticoagulant effect at therapeutic doses required to inhibit cancer growth and spread. The potential of heparin as an anticancer and anti-inflammatory agent led to discovery of a number of low or nonanticoagulant heparins produced by chemical modifications of the heparin polymer itself (Irimura et al., 1986; Lundin et al., 2000; Vlodavsky et al., 1994), sulfation of other natural polymers (Borgenstrom et al., 2007; Kaeffer et al., 1999; Miao et al., 1999) and sulfated heparin-like polymers produced synthetically (Wellstein et al., 1991; Zugmaier et al., 1992). Studies with heparanoids have been limited to animal models. In human studies, heparin has proven beneficial in cancer treatment but its anticoagulant activity has limited its use.

Herein we describe a nonanticoagulant 2-O, 3-O desulfated heparin (ODSH) that retains the anti-inflammatory activities of heparin, including inhibition of P- and L-selectins, heparanase, cationic neutrophil proteases, activation of nuclear factor- κ B (NF- κ B) and

ligation of RAGE by HMGB-1, AGEs and S100 calgranulins (Barry et al., 2010; Lapierre et al., 1996; Rao et al., 2010; Thourani et al., 2000). Also, we describe the first clinical experience with this modified heparin, which has proven safe and low in anticoagulant activity in 137 humans tested to date. Moreover, we present studies demonstrating that ODSH does not cause heparin-induced thrombocytopenia (HIT), a thrombotic syndrome occurring in 3% of individuals receiving heparins (Arepally & Ortel, 2010).

2. Methods

2.1 Production of ODSH

ODSH is a heparin derivative produced for human use under cGMP conditions by the leading U.S. commercial manufacturer of bulk pharmaceutical grade USP heparin, Scientific Protein Laboratories (Wanaukee, WI). It is synthesized by cold alkaline hydrolysis of USP porcine intestinal heparin, as reported (Fryer et al., 1997), which removes the 2-O sulfate groups from iduronic acid residues and the 3-O sulfate groups from the glucosamine residues of the heparin backbone. The N-sulfates, 6-O sulfates, and carboxylates remain intact (Fig.2).

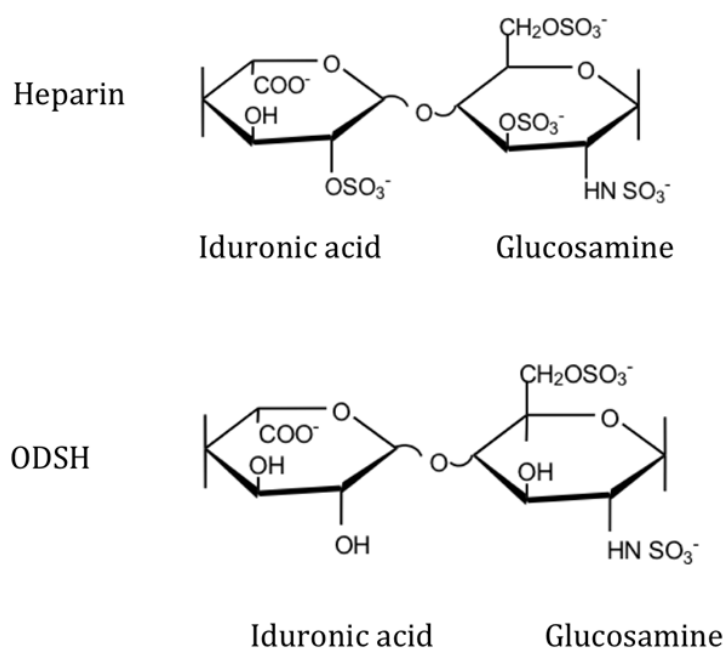


Fig. 2. Sulfate position in disaccharide units of ODSH in comparison to heparin.

2.2 Phase I clinical trials

Two INDs have been opened for intravenous ODSH: #71,356 (under which Phase I safety trials were conducted and pediatric protein losing enteropathy is being studied) and #72,247 (inactive, for a phase II trial in exacerbations of COPD). In the 137 humans who have received intravenous ODSH by bolus and constant infusion for up to 5 days, there have been no serious adverse events, and no decrease in platelet count in any subject to suggest occurrence of HIT, which might have been expected in 2-4 (2-3%) subjects. In Phase I human safety studies (Rao et al., 2010) bolus ODSH was administered safely in doses as high as 20

mg/kg, resulting in lower activated partial thromboplastin times (aPTT) than the usual anticoagulation bolus of unfractionated heparin (80 U/kg, i.e., 0.5 mg/kg at the usual 160 U/mg anti-Xa activity of USP heparin). As part of the Phase I evaluation, ODSH was infused as a bolus of 8 mg/kg followed by continuous intravenous infusion of 0.5 mg/kg/h, adjusted to maintain an aPTT between 40 and 45 seconds (0.64 to 1.39 mg/kg/h final infusion rate).

2.3 Cell culture

U937 monocytes were grown in suspension culture at 37° C in humidified 5% CO₂-95% air in RPMI-1640 supplemented with 10% heat inactivated FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM MEM non-essential amino acids, 100 units/mL penicillin and 100 µg/mL streptomycin. Experiments were performed on cells from passages 1-5. B16F10.1 melanoma cells were grown in Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 100 U/mL penicillin, 100 U/mL streptomycin, 2 mM glutamine and 1 mM sodium pyruvate.

2.4 Adhesion assays

2.4.1 Cell surface binding assays

The effect of heparinoids on binding of U937 monocytes to P-selectin or RAGE was studied in high-bind micro plates coated with 8 µg/mL protein A (50 µL/well) in 0.2 M carbonate-bicarbonate buffer (pH 9.4). Plates were washed with phosphate buffered saline (PBS) containing 1% BSA (PBS-BSA), and P-selectin-Fc or RAGE-Fc chimera (50 µL containing 1 µg) was added to each well and incubated for 2 h at room temperature or overnight at 4° C, respectively. Following incubation, wells were washed twice with PBS-BSA. Fifty µL of heparinoids (0 to 1,000 µg/mL) serially diluted in 20 mM HEPES buffer (containing 125 mM NaCl, 2 mM calcium and 2 mM magnesium) were added to each well and incubated at room temperature for 15 min. As a negative control, 50 µL of 10 mM EDTA was added to select wells to prevent cell binding through sequestration of calcium. At the end of the incubation period, 50 µL of U937 cells (10⁵ cells/well, calcein-labeled according to manufacturer's instructions) were added to each well and plates were incubated an additional 30 min at room temperature. The wells were then washed thrice with PBS, and bound cells were lysed by addition of 100 µL of Tris-Triton X-100 buffer. Fluorescence was measured on a microplate reader using excitation of 494 nm and emission of 517 nm.

2.4.2 Solid phase binding assays

Two types of ELISAs were performed, one to observe the binding between RAGE and its ligands, including CML-BSA, HMGB-1 and S100b, and a competitive ELISA to study the ability of ODSH to inhibit/compete for RAGE binding to its ligands.

To confirm RAGE binding to its ligands, polyvinyl 96-well plates were coated with 5 µg/well of specific ligand (CML-BSA, HMGB-1 or S100b calgranulin). Plates were incubated overnight at 4° C and washed thrice with PBS-0.05% Tween-20 (PBST). Next, 50 µL of RAGE from the dilution series ranging from 0.001 to ~ 6 nM was transferred to each respective ligand-coated well and incubated at 37° C for 1 h. Wells were then washed four times with PBST. To detect bound RAGE, 50 µL of anti-RAGE antibody (0.5 µg/mL) was added to each well, the mixture was incubated for 1 h at room temperature, and wells were washed again four times with PBST. HRP-conjugated secondary antibody (50 µL per well) was added,

wells were incubated for 1 h at room temperature, and then washed once with PBST. A colorimetric reaction was initiated by addition of 50 μ L of TMB and terminated after 15 min by addition of 50 μ L of 1 N HCl. Absorbance at 450 nm was read using an automated microplate reader. Binding affinity (K_D) was determined from the plot of absorbance values versus concentrations of RAGE.

RAGE binding to glycosylated ECM proteins, collagen-I, collagen -IV, fibronectin and laminin coated plates were used. Plates were incubated with 100 μ L of 0.15 M glyoxylic acid + 0.45 M sodium cyanoborohydride mixture at 37 °C for 24 hours. At the end of incubation period, the plates were washed with PBST and used for RAGE binding as described above.

For studies of the effect of ODSH or heparin on RAGE binding to its ligands, polyvinyl 96-well plates were coated with specific ligand as described above. Separately, a constant amount of RAGE-Fc chimera (100 μ L containing 0.5 μ g/mL in PBST containing 0.1% BSA) was incubated with an equal volume of serially diluted ODSH or heparin (0.001 to 1,000 μ g/mL in PBST-BSA) overnight at 4° C. The following day, 50 μ L of RAGE-heparinoid mix was transferred to each respective ligand-coated well and incubated at 37° C for 2 h. Wells were then washed four times with PBST. Bound RAGE was detected as described above. Absorbance at 450 nm was plotted against concentration of ODSH or heparin. The IC_{50} values were obtained using non-linear regression analysis.

2.5 Elastase and cathepsin G activity assay

The inhibitory activity of heparin and ODSH against HLE and cathepsin G was monitored using the specific chromogenic substrates Suc-Ala-Ala-Val-*p*NA and Suc-Ala-Ala-Pro-Phe-*p*NA, respectively, according to methods previously described (Fryer et al., 1997).

2.6 Mouse model of melanoma lung metastasis

Lung metastasis from melanoma was studied using protocols previously reported by the Varki group (Stevenson et al., 2005). Animal use and husbandry followed protocols approved by the IACUC at the University of Utah. Confluent B16F10 melanoma cells (70-80%) were harvested by brief exposure to trypsin, and washed twice with serum-free medium prior to injection. Living cells were counted with Trypan blue staining prior to injection to insure > 95% viability. Female C57BL/6J mice (n = 6 per group) were injected subcutaneously with 100 μ L PBS, heparin (30 mg/kg) or ODSH (30 mg/kg). Thirty min later, 5×10^5 B16F10 cells in 200 μ L medium were injected intravenously into the lateral tail vein. Mice from each group were injected in alternating order, and cells were resuspended by gently flicking the tube before aspirating the sample for each injection. Twenty-seven days after injection, surviving mice were euthanized. The lungs were removed, perfused intra-tracheally with 10% buffered formalin and photographed. Visible tumor foci were counted independently by two different laboratory personnel blinded with regard to treatment groups, and metastasis quantified in terms of the number of black spots.

3. Results and discussion

3.1 ODSH is a non-anticoagulant heparin that is manufactured at industrial scale and safe from heparin induced thrombocytopenia

The ODSH was manufactured as described in Methods section. Seven serial 1-2 kg batches of material have shown an average molecular mass of 11.7 ± 0.3 kg kDa. ODSH has low affinity for anti-thrombin III (K_D = 339 μ M or 4 mg/mL vs. 1.56 μ M or 22 μ g/mL for

heparin) consistent with its low anticoagulant activity. Serial batches of ODSH demonstrated consistently reduced USP (7 ± 0.3 U of anticoagulant activity/mg), anti-Xa (1.9 ± 0.1 U/mg), and anti- IIa (1.2 ± 0.1 U/mg) activities, compared with those of heparin (165-190 U/mg activity for all 3 assays). The current clinical formulation that was used in 137 humans (54 normal and 83 ill patients) is a sterile-filled 20 mL glass vial containing an isotonic 50 mg/mL solution of ODSH in buffered saline. This formulation is cGMP manufactured by Pyramid Laboratories (Costa Mesa, CA). Chemistry and Manufacturing processes are already on file with the FDA in two INDs. A complication of heparin is HIT, which occurs in subjects who produce an activating antibody to the heparin-platelet factor 4 (PF4) complex on the platelet surface, causing thrombocytopenia and thromboembolism (Arepally & Ortel, 2010). In standard serotonin release assays (SRA) performed with human antibodies to the heparin-PF4 complex, heparin triggered release of ^{14}C -serotonin from normal platelets (Fig 3), but ODSH did not trigger HIT at any concentration (Rao et al., 2010).

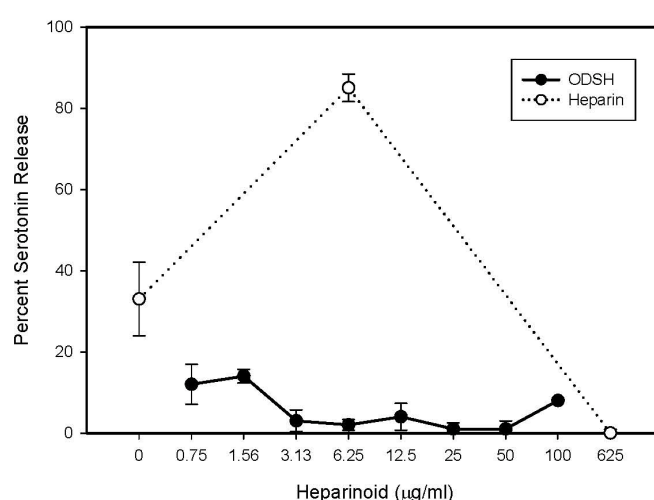


Fig. 3. ODSH does not trigger platelet activation in the serotonin release assay. Normal platelets were loaded with ^{14}C -serotonin and incubated with serum from 3 patients with heparin-PF4 antibodies. Platelets were activated by heparin as a positive control ($\geq 20\%$ serotonin release). Rao et al., Am J Physiol Cell Physiol (2010) Am Physiol Soc, used with permission.

3.2 ODSH is safe to use in humans

Two INDs have been opened for intravenous ODSH: #71,356 (under which Phase I safety trials were conducted and pediatric protein losing enteropathy is being studied) and #72,247 (inactive, for a phase II trial in exacerbations of COPD). In the 137 humans who have received intravenous ODSH by bolus and constant infusion for up to 5 days, there have been no serious adverse events, and no decrease in platelet count in any subject to suggest occurrence of HIT, which might have been expected in 2-4 (2-3%) subjects (Arepally & Ortel, 2010). In Phase I human safety studies (Rao et al., 2010) bolus ODSH was administered safely in doses as high as 20 mg/kg, resulting in lower activated partial thromboplastin times (aPTT) than the usual anticoagulation bolus of unfractionated heparin (80 U/kg, i.e., 0.5 mg/kg at the usual 160 U/mg anti-Xa activity of USP heparin). As part of the Phase I evaluation, ODSH was infused as a bolus of 8 mg/kg followed by continuous intravenous

infusion of 0.5 mg/kg/h, adjusted to maintain an aPTT between 40 and 45 seconds (0.64 to 1.39 mg/kg/h final infusion rate). This protocol achieved anti-inflammatory plasma ODSH concentrations (≥ 10 -20 $\mu\text{g/mL}$) at doses that did not cause anticoagulation (Fig 4).

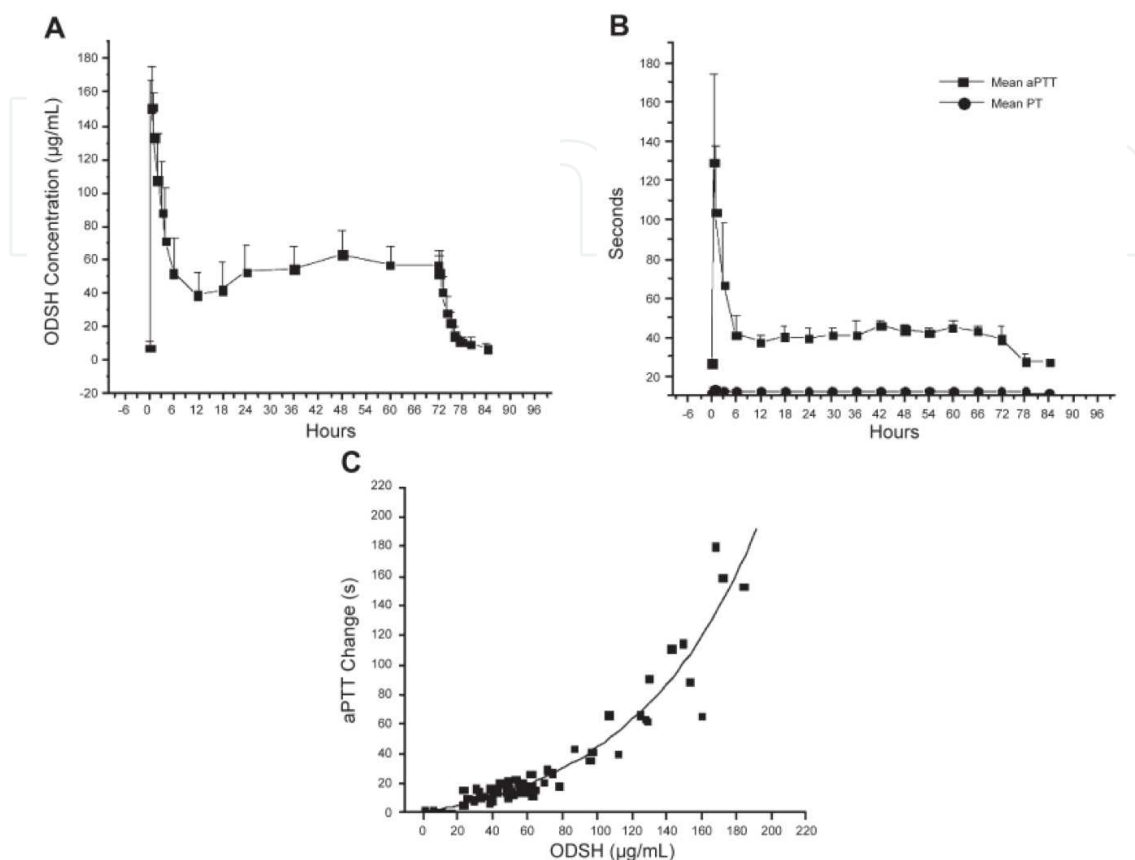


Fig. 4. ODSH infusion in human volunteers achieves anti-inflammatory concentrations before producing anticoagulation. A. Mean (\pm SD) plasma ODSH levels during study drug infusion, measured by an automated GLP validated assay (Rao et al., 2010). B. Mean (\pm SD) aPTT and change in prothrombin time (PT) during study drug infusion. C. Relationship between plasma ODSH and change in aPTT from baseline during the 3 day infusion of 8 mg/kg followed by 0.5 mg/kg/h adjusted to maintain aPTT between 40 and 45 seconds. Rao et al., *Am J Physiol Cell Physiol* (2010) Am Physiol Soc, used with permission.

3.3 ODSH has anti-metastatic and anti-inflammatory activities in vitro

Despite low anticoagulant activity, ODSH retains the strong, broad anti-inflammatory activities and antimetastatic activities of heparin. In metastasis, cancer cells disseminate into the blood circulation and interact with other circulating cells like leukocytes and platelets and also endothelium of vascular wall by selectin-mediated adhesion. The family of selectins includes, P-, L- and E-Selectin, which are expressed on activated platelets, leukocytes and endothelium. Tumor cells express mucins, which are the ligands for selectins, on the cell surface and facilitate cell-cell adhesion. The involvement of selectins in metastasis has been shown in selectin-deficient mouse models (Borsig et al., 2002; Kim et al., 1998; O et al., 2002). Several studies have shown that heparin and heparinoids inhibit selectin-mediated cell adhesion, an important phase of metastasis (Borsig, 2010; Borsig et al., 2001; Borsig et al., 2002; Gao et al., 2006; Ma & Geng, 2000; Maraveyas et al., 2010; Stevenson

et al., 2005; Stevenson et al., 2007; Varki, 2007; Wahrenbrock et al., 2003; Wei et al., 2004). To investigate the antimetastatic potency of ODSH, we used U937 cells, a human monocytic leukemia cells. U937 cells express P-selectin ligand, P-selectin glycoprotein ligand-1 (PSGL-1), that functions similar to mucins. We tested the ODSH ability to competitively displace fluorescent-labeled U937 cells adherence to P-selectin via PSGL-1. ODSH inhibited P-selectin with an IC_{50} of 1.1 $\mu\text{g}/\text{mL}$ consistent with that of values reported by Wang (Wang et al., 2002), who demonstrated that ODSH also inhibits L-selectin-mediated adhesion with an IC_{50} of 3 $\mu\text{g}/\text{mL}$, versus 0.4 $\mu\text{g}/\text{mL}$ for heparin (Wang et al., 2002) (Fig. 5).

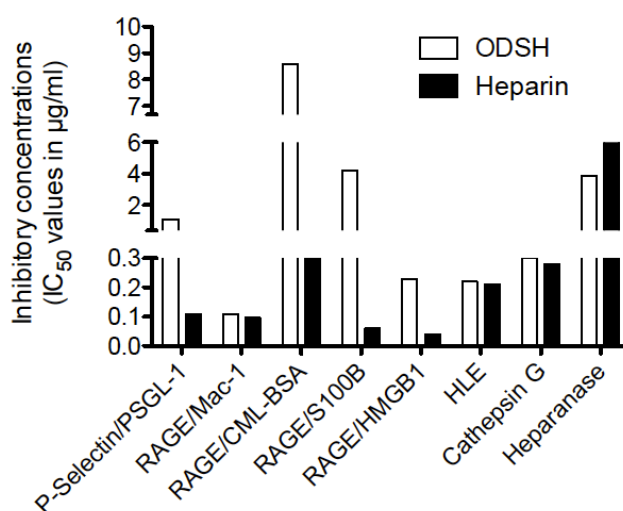


Fig. 5. Anti-inflammatory and anti-metastatic profile of ODSH. The 50% inhibitory concentrations (IC_{50} values) of ODSH to interrupt the receptor ligand pairs, P-selectin/PSGL-1, RAGE/Mac-1, RAGE/CML-BSA, RAGE/S100B and RAGE/HMGB1, interaction are higher than that of heparin. Nonetheless, the IC_{50} concentrations of ODSH required to inhibit HLE, cathepsin G and heparanase activities, the key enzymes involved in the inflammation and metastasis, are similar to the values for heparin. The IC_{50} values of ODSH are higher than that of heparin suggesting that ODSH has lower inhibitory potential than heparin, but the values are much lower than achievable plasma concentration ($\sim 200 \mu\text{g}/\text{mL}$) in humans (see Fig 4). Heparanase data is from (Lapierre et al., 1996).

Though RAGE binds to disparate ligands, we will discuss only the ligands AGE, HMGB1 and S100B that have been evaluated for their role in cancer. RAGE and its ligands are co-expressed in many tumors and the expressed ligands are secreted causing triggering of cellular signaling pathways. This results in expression of cytokines, growth factors, transcription factors, adhesion molecules and NF- κ B (Sparvero et al., 2009). Therefore we will focus on the interruption of RAGE-ligand interaction with ODSH.

Several in vitro studies have shown that AGE stimulates growth, proliferation and invasion of cancer cells including melanoma cells (Abe et al., 2004; Yamamoto et al., 1996; Zill et al., 2003; Zill et al., 2001). AGEs are found in extracellular matrix (ECM) proteins (Ling et al., 1998; Mizutani et al., 1997) that can interact with RAGE on tumor cells and enhance RAGE expression causing damage to surrounding tissues. We therefore tested the binding of native and glycosylated ECM proteins (collagen I, collagen IV, fibronectin and laminin) to RAGE. Results indicate that RAGE binds to native ECM protein with a strong affinity but the affinity is negligibly decreased with glycosylated ECM proteins. Overall, the K_D values of

RAGE for different ECM proteins are less than 100 ng/mL or <1nM suggesting RAGE on tumor cells might play a role in tumor cell spreading (Fig 6).

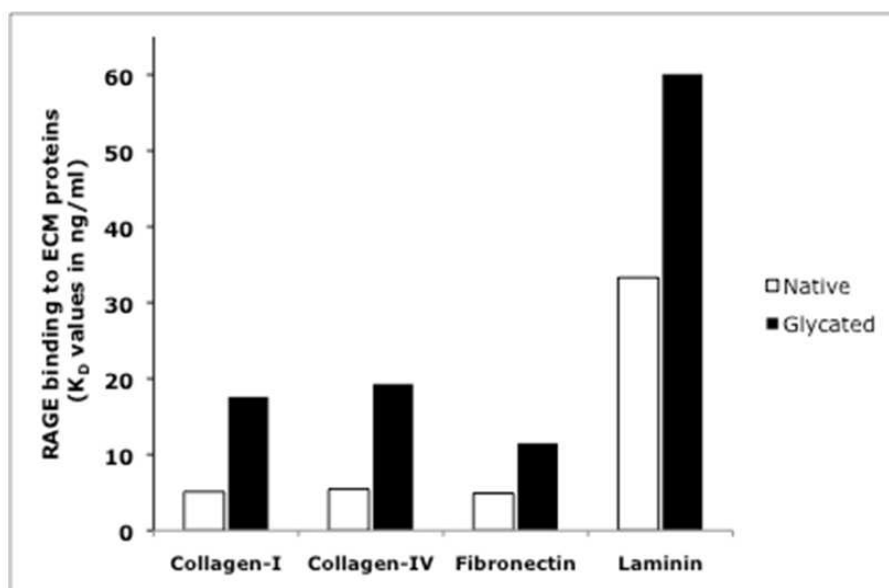


Fig. 6. RAGE binds to AGE-ECM proteins. RAGE binding to ECM proteins was analyzed as described in Methods Section. RAGE bound to native Collagen I, Collagen IV and fibronectin with same affinity but with a lesser affinity to Laminin. The affinity of RAGE for glycated ECM decreased for all the ECM proteins studied but the affinity constants suggest that RAGE binds strongly to ECM proteins even in modified conditions.

In addition, AGE-CML is a common AGE found in vivo (Reddy et al., 1995) and has been detected in tumor tissues (van Heijst et al., 2005). Therefore, we tested potential of ODSH to disrupt the binding between CML-BSA and RAGE as described in methods section. We observed that RAGE bound to CML-BSA with strong affinity (K_D 0.43 nM) and ODSH inhibited the ligation of CML-BSA with IC_{50} of 8.6 μ g/mL (Fig 5).

HMGB1 is another ligand for RAGE and both HMGB1 and RAGE have been shown elevated in tumors, including melanoma, colon, prostate, breast and pancreatic cancers (Brezniceanu et al., 2003; Ellerman et al., 2007; Sparvero et al., 2009). Earlier studies by Huttunen et al. suggested that RAGE/HMGB1 signaling might be associated with metastasis (Huttunen et al., 1999; Huttunen et al., 2000). Taguchi et al. found that blockade of HMGB-RAGE signaling suppressed tumor growth as a result of decreased cell migration and invasiveness rather than proliferation or apoptosis (Taguchi et al., 2000). HMGB1 alone or by interaction with NF-kBp65 has been shown to bind specific region of melanocyte inhibitory activity (MIA) promoter in melanocytes and increase the expression of MIA protein, which enhances migration and invasion of tumor cells (Poser et al., 2003; Sasahira et al., 2008). HMGB1 was isolated using heparin-sepharose chromatography (Rauvala & Pihlaskari, 1987) and later was identified to contain a heparin-binding sequence in its amino terminus, suggesting its affinity for heparin (Cardin & Weintraub, 1989). Consistent with these observations, HMGB1 also interacted with carbohydrate chain of syndecan and facilitated cell migration (Rauvala & Rouhiainen, 2010). Syndecan is expressed in normal tissues and tumor tissues but the role of HMGB1 interaction with syndecan in physiological

and pathological conditions is not known (Rauvala & Rouhiainen, 2010). Since ODSH is derived from heparin, we tested whether ODSH can interrupt the RAGE-HMGB1 ligation. Though HMGB1 binds to RAGE with high affinity (K_D 0.64 nM), ODSH inhibited RAGE-HMGB1 interaction with an IC_{50} of 0.23 $\mu\text{g/mL}$ (Figs. 5 and 7).

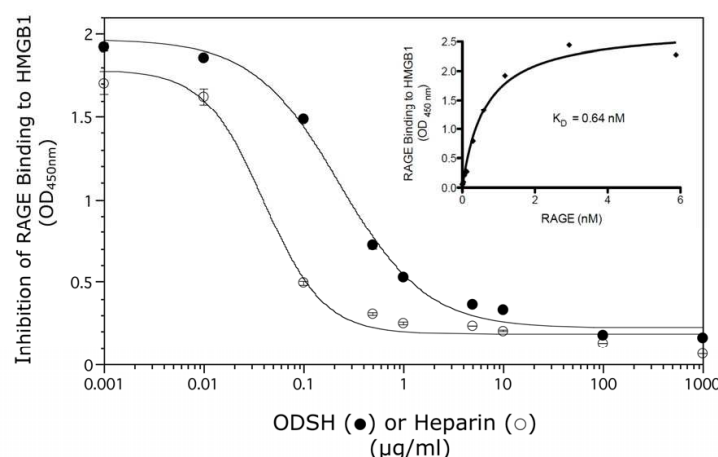


Fig. 7. ODSH and heparin inhibit binding of HMGB-1 to RAGE. HMGB-1 binding to RAGE-Fc chimera was studied using ELISA techniques as described in Methods. RAGE bound to immobilized HMGB-1 in a saturable fashion (*inset*) with a K_D of 0.64 nM. ODSH (●) and heparin (○) inhibit binding of recombinant HMGB-1 to RAGE with IC_{50} values of 0.23 and 0.04 $\mu\text{g/mL}$, respectively. Rao et al., Am J Physiol Cell Physiol (2010) Am Physiol Soc, used with permission.

S100 proteins are calcium dependent proteins of the EF-hand (helix-loop-helix) type that are differentially located and differentially expressed in a wide variety of cells (Donato, 1999; Donato, 2001; Donato et al., 2009). Among the 25 S100 protein identified, S100b is unique in its location on chromosome 21q22.3 and first one of the group found to ligate to RAGE (Hofmann et al., 1999). S100b is highly expressed in melanoma and considered as possible biomarker for the prognosis of the disease (Harpio & Einarsson, 2004; Salama et al., 2008). We studied the binding affinity of S100b to RAGE. RAGE ligated to S100b with a K_D of 0.45 nM, a value much lower than the affinity range reported (reviewed in (Leclerc et al., 2009)). ODSH inhibited the binding of RAGE to S100b with IC_{50} value of 4.23 $\mu\text{g/mL}$. (Fig 5). HMGB-1 is released into extracellular milieu as a result of necrosis, apoptosis and secretion. Only HMGB1 released by necrotic cells demonstrates cytokine activity, recruits leukocytes and activates endothelial cell adhesion molecules such as selectins (Dumitriu et al., 2005; Lotze & Tracey, 2005). In cancer, tumor cells undergoing stress are susceptible to death because of factors that include hypoxia, nutrient deprivation and or anticancer treatments (Kepp et al., 2009). These dying cancer cells become a source of HMGB1 (Dong Xda et al., 2007; Scaffidi et al., 2002). Orlova et al (Orlova et al., 2007)) have shown that HMGB1-induced Mac-1 dependent neutrophil recruitment requires the presence of RAGE on neutrophils but not on endothelial cells. Interestingly, three proteins, HMGB1, Mac-1 and RAGE bind to heparin (Diamond et al., 1995; Hanford et al., 2004; Rauvala & Pihlaskari, 1987). We therefore tested Mac-1 dependent binding of U937 cells to immobilized RAGE. ODSH inhibited the adherence of U937 cells to RAGE with IC_{50} values of 0.11 $\mu\text{g/mL}$ (Fig 5).

3.4 ODSH Prevents metastasis in vivo

The *vitro* studies described above suggested that ODSH, similar to heparin, might interrupt key receptor-ligand interactions involved in metastasis. We therefore investigated the effects of ODSH in experimental melanoma lung metastasis. Heparin significantly decreased lung metastasis at 28 days after melanoma injection, but the same dose of ODSH provided substantially greater reduction of lung metastasis (Fig 8A). ODSH also significantly improved 28-day survival. In contrast to 70% mortality in mice treated with PBS, ~ 70% of ODSH-treated mice survived the experiment (Fig 8B).

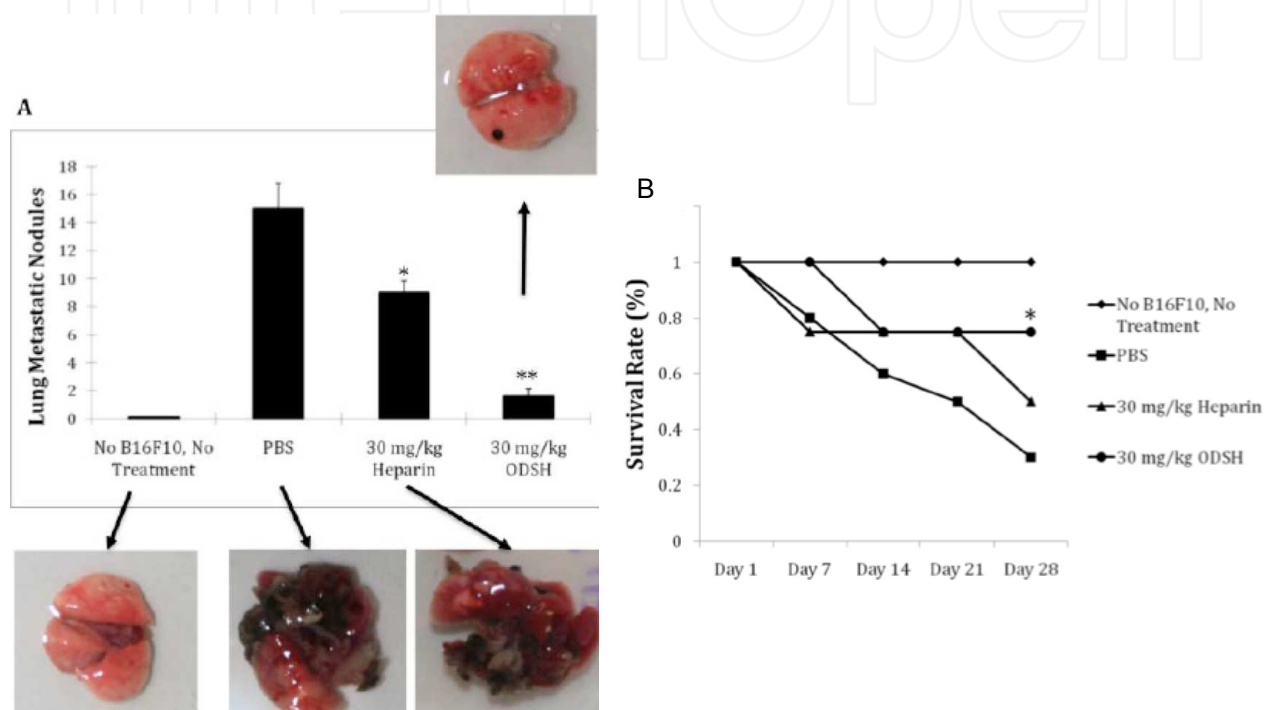


Fig. 8. ODSH inhibits melanoma lung metastasis. Female C57BL/6J mice ($n = 6/\text{group}$) were injected SQ with 100 μL PBS, 30 mg/kg heparin, or 30 mg/kg ODSH. Thirty min later, 5×10^5 B16F10 melanoma cells were injected IV into the tail vein. 27 days later surviving mice were euthanized and lungs were removed, fixed, stained and assessed for metastasis by 2 independent observers. A. Heparin ($*P < 0.05$ vs PBS) or ODSH ($**P < 0.01$ vs PBS) significantly reduced metastasis. B. SQ heparin showed little effect on lung metastatic outgrowth but ODSH suppressed metastatic colonization microscopically. C. ODSH improved survival ($*P < 0.05$ vs PBS). Rao et al., Am J Physiol Cell Physiol (2010) Am Physiol Soc, used with permission.

This model is widely used to test the inhibitory capacity of heparin and heparins with no or minimal anticoagulant activity in metastasis (reviewed in (Borsig, 2010)). The attenuation of metastasis was attributed to P-selectin and L-selectin as the experimental mice were deficient in selectin but also suggested that there is a selectin-independent mechanism involved. It is not clear whether B16 melanoma cells express mucins on the cells surface to interact with P-selectin, but the expression of RAGE is observed in melanoma cells (Huttunen et al., 2002; Saha et al., 2010). Further, Huttunen et al showed the attenuation of metastasis when B16 cells injected with HMGB1 peptide suggesting the RAGE-mediated tumor cell invasion can be inhibited by competition with RAGE binding ligand such as

ODSH and heparin. In summary, because ODSH interacts with the many receptors, ligands and inhibits enzymes involved in the metastasis, the attenuation of metastasis in the experimental model is due to action of ODSH on multiple sites.

4. Conclusion

A number of fully anticoagulant heparins are commercially available, but their use as a chronic treatment for cancer is dose-limited by their anticoagulant activity. Therapeutic anticoagulant doses of heparin or low molecular weight heparin (Hirsh et al., 2001; Koenig et al., 1998) do not provide sufficiently high plasma drug concentrations to reliably inhibit the selectin-, heparanase- or RAGE-mediated processes (Koenig et al., 1998; Lapierre et al., 1996; Rao et al., 2010; Wang et al., 2002) that are now understood to be important for explaining the benefits of heparins in cancer.

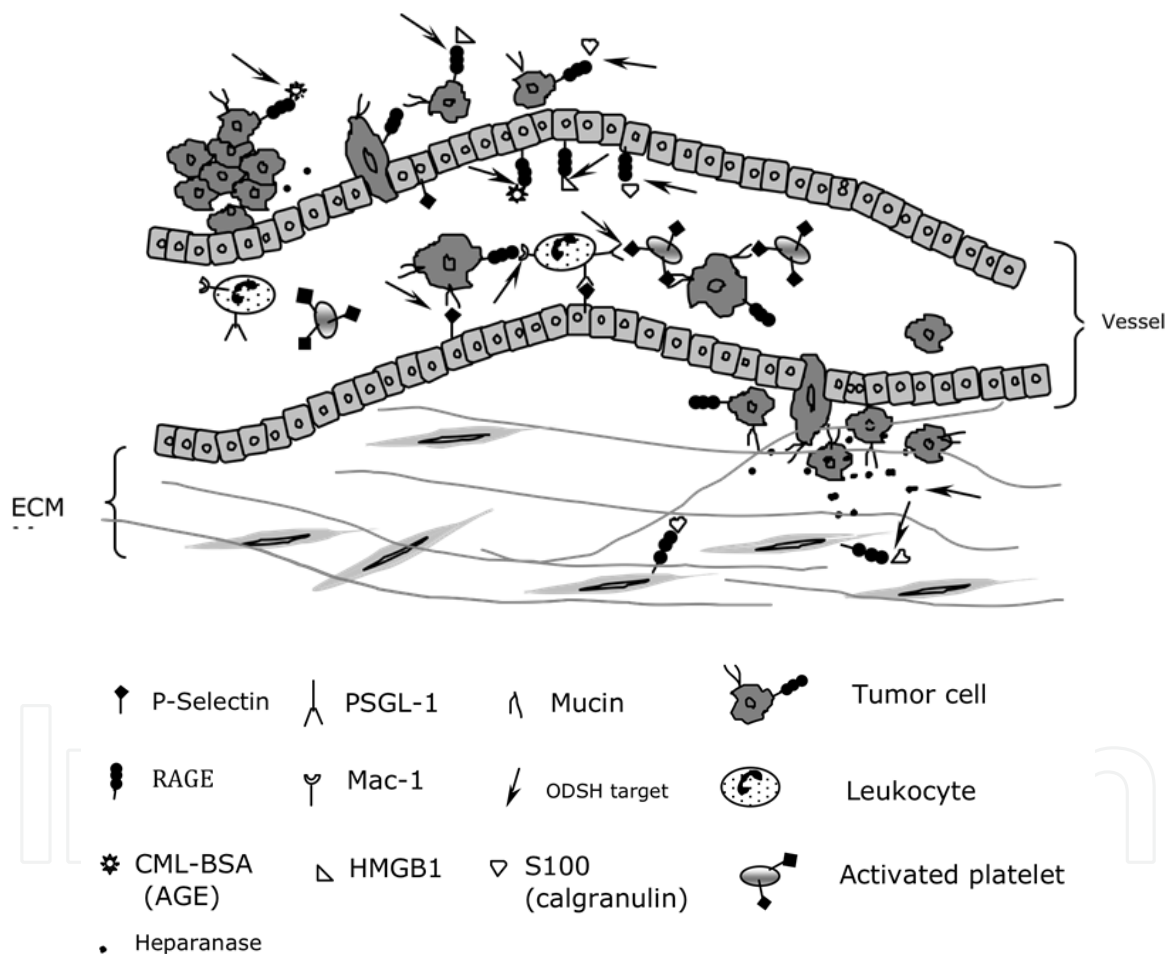


Fig. 9. Potential interference of low anticoagulant ODSH in metastasis. ODSH 1) inhibits heparanase, the enzyme secreted by tumor cells during intravasion and extravasation thus prevent spread of the tumor cells, 2) inhibits the tumor cell mucin (P-selectin ligand) binding to P-selectin on endothelial cells and platelets, 3) inhibits tumor cell RAGE interaction with leukocyte Mac-1, 4) inhibits tumor cell RAGE interaction with its ligands, AGE, HMGB1 and S100b, that are either released by tumor cells within cancer micro environment or by the leukocytes thus prevent sustained signaling for tumor progression, 5) inhibits circulating RAGE ligand interaction with endothelial RAGE.

Of the available low anticoagulant heparin derivatives (Casu et al., 2008; Fryer et al., 1997; Kragh et al., 2005; Kragh & Loechel, 2005; Lapierre et al., 1996; Ono et al., 2002; Rao et al., 2010; Stevenson et al., 2007; Wang et al., 2002; Yoshitomi et al., 2004), only ODSH has been proven safe from major adverse events in humans (Rao et al., 2010) data on file with FDA) and only ODSH is free from the potential to induce HIT (Rao et al., 2010). The concept that P-selectin is the key molecule in metastasis is based on the attenuation of metastasis in animal models. Because of the complex biology of cancer metastasis, it is not easy to identify the specific mediator(s) involved in development of metastatic disease in patients with cancer. In such situation, a compound that can simultaneously target multiple mediators involved in the diseases might have substantial therapeutic value. We have described that non-anticoagulant ODSH, not only targets P-selectin, but additionally is capable of inhibiting the RAGE interaction with its multiple structurally unrelated ligands that play many biological roles in metastasis (Fig. 9). Thus, the results described herein support the notion that low anticoagulant heparin derivatives such as ODSH might prove useful in prevention of metastasis in human cancer.

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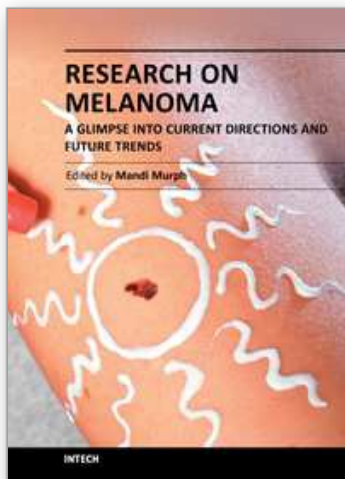
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The book *Research on Melanoma: A Glimpse into Current Directions and Future Trends*, is divided into sections to represent the most cutting-edge topics in melanoma from around the world. The emerging epigenetics of disease, novel therapeutics under development and the molecular signaling aberrations are explained in detail. Since there are a number of areas in which unknowns exist surrounding the complex development of melanoma and its response to therapy, this book illuminates and comprehensively discusses such aspects. It is relevant for teaching the novice researcher who wants to initiate projects in melanoma and the more senior researcher seeking to polish their existing knowledge in this area. Many chapters include visuals and illustrations designed to easily guide the reader through the ideas presented.

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