Beyond Anticoagulant: Heparin as a Potential Anti-cancer Agent

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INTRODUCTION

The well known anticoagulant compound heparin was discovered in the early 20th century by Howell’s and his student L. Emmett Holt Jr. Howell coined the term heparin from the Greek ‘hepar’ meaning liver, the tissue utilized in the isolation of heparin [1]. The family of glycosaminoglycan (GAGs) consists of sulfated and non-sulfated GAGs. The sulfated class of GAGs is further subdivided into O-sulfated and N-sulfated GAGs. The heparin and long chain less sulfated polymer known as heparan sulfate (HS) comes under N-sulfated GAGs category. The presence of heparin with its similar structure has been noticed in many vertebrate and invertebrate organisms [2] including turkey [3], whale [4], camel [5], mouse [6], human [7], losbster [8], shrimp [9], mussel [10], species of clam [11] and crab [12].

The biochemical characteristic that differentiate HS to heparin is basically based on the sulfation pattern, as HS is less sulfated than heparin and possesses higher level of acetylation on its glucosamine residue. Heparin is solely produced by the mast cells of connective tissues in the form of serglycin, a proteoglycan (PG). The complex procedure of biosynthesis of heparin in mast cells involves substantial level of sulfation with epimerization of its uronic acid. The whole process takes place in such a way that more than 80% of the glucosamine can be changed into deacetylated form with N-sulfation. The process converts 70% of the uronic acid into iduronic acid. HS on the other hand exist in association with its PG, the basic structure of HS is similar to heparin but bears lower degree of epimerization to its iduronate and also with lower level of N- and O-sulfation and charge density along the polymer [13]. The major sequence of heparin comprises of iduronate residues, often possesses sulfation at C2 positions with a N-sulfated glucosamine. Apart from sulfation at nitrogen moiety, the amino sugar part (glucosamine) is also known to have sulfation at the 6-O position. Variation in the pattern of sulfation and its distribution provides substantial heterogeneity, which can affect its biological properties [14]. The main biological property for which heparin is known all over is its ability to act as an anti-coagulant. The strong anti-coagulant property of heparin is mainly because of its ability to potentiate the anti-thrombin
There are numbers of affinity states between heparin and AT with its coagulation factors which ultimately terminates into high affinity interactions. There are two possible mechanisms by which heparin are known to activate AT. The first is altering the structure of AT which allows further interaction between heparin and AT results in stronger binding between these two. The conformational change also hinders the protease reactive centre loop (RCL) in AT. This alteration in structure allows interaction of AT with factor Xa. After complex formation, the ATIII bounces back to its original low affinity binding state, which results in cleavage of RCL and liberation of heparin from the covalent complex of ATIII and factor Xa. The second mechanism of heparin anticoagulation activity is known as bridging mechanism by which a complete heparin molecule facilitate the binding of AT with thrombin. In this mechanism, a positively charge thrombin domain binds non-selectively with extended polymer of heparin. The length based interaction of heparin with serine proteases and AT has already been discussed in several studies, despite the fact that only pentasaccharide portion of heparin is required to interact with AT but a chain of at least 16 monosaccharides are required to initiate the interaction of AT with thrombin [15].

Besides of its known anticoagulant activity, heparin exhibits several other pharmacological activities due to its capacity to interact with diverse proteins. In addition, the presence of carboxylate and sulfate group endows it to possess a high negative charge (approximately -75), which allows it to have electrostatic interaction with many proteins such as growth factors, protease and chemokines [16]. Apart from its anticoagulant activity, the anti-tumor activity of heparin is gaining continuously a substantial ground in upcoming studies, due to its inhibitory effect on vascular thromboembolism and on the pathway of angiogenesis. Now-a-days researchers are focusing on generating short fractions heparin known as low-molecular-weight heparins (LMWHs) sequence which lack anticoagulant property but have more anti-metastatic effect allowing the sequence to bind with tumor growth driving proteins like P-Selectin and fibroblast growth factors [17]. This review will focus on the light molecular weight heparins and their anticancer potential.

**Light molecular weight heparin as anticancer**

Generally, regular fraction of heparin extracted from pork intestine or bovine lung lies between the molecular weights of 5,000 and 40,000 Daltons. Low-molecular-weight heparin is depolymerized form of unfractionated heparin comprises of heterogeneous sulfated group and are generally used to treat venous thromboembolism. The anti-tumor activity is due to its potential to inhibit tumor growth, which results improvement in survival rate [18]. There are different possible mechanisms behind this activity of heparin. One of them is the triggering the coagulation cascades or their other components like tissue factors which are instinctively involve in tumorigenesis and metastasis [19-20]. Several in-vivo studies suggests that’s its antimestatatic role is probably due to its anticoagulant effect, blocking of heparanase, intervention with P-selectin-HSPG interaction and inhibitory effect on tumor adhesion and motility [21]. Dargo et al. in their study demonstrated that heparin and HS decrease cancer metastasis in the Nb rat prostate adenocarcinoma model [22]. The new low-molecular-weight heparin (LMWH) known as revaparin not only block collaged adhesion and adenocarcinoma cells invasion of matrigel but also decrease their intra-abdominal growth in vivo [23]. Many cell-based studies have demonstrated that LMWH can inhibit angiogenesis in a dose-dependent manner, while animal studies have shown that LMWH can alter tumor progression and restrict pulmonary metastasis [24-25]. The phase 2 clinical trial of dalteparin another member of LMWH has showed a promising effect in reducing ovarian cancer at a dose of 100 IU/kg [26]. In terms of clinical research, both warfarin and heparin have been tried to investigate the influence of antithrombotics on cancer. However, more detailed research has been conducted on LMWH. The first potential anticancer effect of antithrombotics was reported in 1954 [27]. The first trial of ultra-fractionated heparin (ULH) was carried out on 277 patients, to estimate the action of ULH on the survival of small cell lung carcinoma [28]. The patients were randomized to receive chemotherapy alone or chemotherapy with dose adjusted subcutaneous UFH for 5 weeks. The group treated with UFH, attained better median survival of 317 days in comparison to 261 days of chemotherapy alone group and also with a better survival rate at 1 year (40 vs 30%), 2 year (11vs 9%), and 3 year (9 vs 6%). The preliminary data to suggesting the effect of LMWH in improving the survival of cancer patient is basically originated from an evaluation studies comparing UFH with LMWH in the first phase of thrombosis treatment [29-30]. However, the first study designed to know the effect of LMWH on survival rate in cancer was named as Fragmin Advanced Malignancy Outcome Study (FAMOUS) study in which 385 patients with lung, pancreatic, hepatic, advanced breast, ovarian, urogenital or uterine cancer were randomized to classical chemotherapy with 5000 IU daily dose of dalteparin or placebo [31]. The primary end point was mortality at one year. The estimate of 1 year survival in the dalteparin and placebo cases were 46 and 41%, respectively, for 2nd year the survival was 27 and 18% and in the 3rd year it was 21 and 12% for dalteparin and placebo group. A post hoc analysis of patients who survived more than 17 months indicated a survival benefit for dalteparin group.

The perceptible advantage of LMWH with an improved prognosis was again shown in another trial having patients with metastatic or locally advanced cancer who could not be eradicated curatively [32]. In this, 302 patients were randomized to receive their regular chemotherapy with a 6-week regime of weight adjusted nadroparin or placebo. Unlike dalteparin, here the primary end point was all-cause mortality. At the period of 6 month, the survival estimates were 61% in nadroparin group and 56% in placebo. At 12 and 24 months the relative estimates were 39 vs 27% and 21 vs 11%. In contrast to these studies, another study with a randomized 141 patients going through the advanced stage of cancer treated with LMWH or saline does not produce any significant survival advantages with LMWH [33]. This shows that LMWH cannot act as anti-tumor alone until incorporated with chemotherapy regime. Due to unsatisfactory response, the saline part was removed from the study to make it more realistic by comparing LMWH with standard care. The median survival reported for standard care was 10.5 months in comparison to 7.3 months with LMWH. Another study on 84 small cell lung carcinoma patients evaluated the efficacy of dalteperin with and without chemotheraphy [34]. The study reported high overall response rate in patients, received LMWH with chemotherapy as the survival rate was 69.2% in comparison 42.5% solely by chemotherapy. A systematic study and comprehensive analysis of all these studies suggests that LMWH with chemotherapy produced better survival in cancer patients including those patients who are at advance stage of cancer [35-36].
Proposed mechanism of action

Inhibition in tumor progression due to anticoagulant property

The exact mechanism of LMWH anti-metastatic role is under scrutiny but as per the animal models and cell based research, some evidences have came into light which relates its anti-metastatic activity to its anti-coagulant activity. Clinically, the LMWH and un-fractionated heparin has been used from a long time as effective blocker of fluid phase coagulation by enhancing antithrombin inactivation of factor IIa and Xa. [37-39] Another anti-metastatic activity of heparin is mainly by the virtue of its anticoagulant effect and is its potential to liberate tissue factor pathway inhibitor (TFPI) from vascular endothelium. The TFPI is one of the contributors in antiangiogenic activity [40-41]. Various studies have demonstrated the effect of antitmetastic activity of modified heparin with no anticoagulant activity [42-47]. These modified heparins ruled out the claim that antitmetastic activity is due to anticoagulant effect of heparin. In another study, an excellent anticoagulant agent, fondaparinux that mainly potentiate heparinase activity [66-70], had produced no effect on cancer progression at clinically acceptable dose [48-49].

Inhibition of heparinase

Another important step in the process of cancer progression, which drive the invasion of tumor cells is the debasement of various building blocks of extracellular matrix, including laminin, collagen, fibronectin and HSPGs. Cancer cells usually get this job done by employing some hydrolytic enzymes such as matrix metaloproteases, serine proteases, cysteine proteases and endoglycosidases [50-51]. Among endoglycosidases, heparinases secreted by tumor cells can sabotage various component favoring tumor invasions. The expression of heparinase is very rare in normal tissues but its expression is evident in many tumors where it significantly enhances both metastasis and angiogenesis [52] of tumors of breast [53], colon [54], ovary [55], bladder [56], pancreases [57], acute myeloid leukemia [58], non-small cell lung cancer [59] and myelomas [60]. Heparinase is a vital enzyme for the cleavage of heparin sulfate groups present in heparan sulfate proteoglycans. These HS groups possess growth factor having domain associated to the protein core with serine residue confined in ECM and in plasma membrane of cells. These domains engage growth factors, like bFGF and VEGF [61-62] and then act as their co-factor in cell signaling [63-61]. Heparinase not completely cleaves the heparan sulfate chain but it breaks the glycosidic bonds at some specific sites, generating sequences, which seems even more potent than the parent chain in activation of bound growth factors [64-65]. Various study demonstrated that heparin and some chemically altered forms of heparin blocked the tumor cell heparanase activity [66-70]. Thus, inhibiting heparinase can subsequently reduce metastatic potential of tumor [71].

Engagement with cell adhesion molecules

Invasion of tumor cells from one part of body to other part usually takes place through extravasation capability of tumor cells by which they invade blood vessels and reach to distant organs. The extravasation phase is also critical step for tumor cells from the prospect of their survival in blood vessels. To survive against the immune competent cells present in stream of blood, the tumor cells form a complex with platelets [72-73] which not only provide a cover that protect them against immune system elements but also support their anchoring on vascular endothelium [74]. This complex formation between tumor cells and platelets is mediated by interaction of glycoprotein’s in the plasma membrane of tumor cells to the selectins of platelets and endothelium.

Transformed glycosylation of cell-surfaced mucins is a leading feature of tumor growth. Some of these transformations are related with carcinomas (Cancer originate from epithelial cells). Sialyl Lewis (a) and Lewis x are two of such epitopes found on the carcinoma mucins that are usually associated with tumor progression. Selectins act as adhesion receptors that recognize these altered glycosylated structures. Their physiological role in facilitating cell adhesion has already been demonstrated in inflammations, immune responses and wound repairs [75-76]. Selectins are basically adhesion molecules responsible for initiating the first step for cell adhesions and in the absence of selectins, all the steps are initiated by secondary elements of adhesion process such as integrins and other adhesion molecules are eventually delayed or do not occur. Selectins are expressed by leukocytes (L-selectin), platelets (P-selectin), and the vascular endothelium (E and P selectin). However, L selectin mainly exist on neutrophils, monocytes, and naïve lymphocytes. The secretory granules of resting platelets and endothelium stores P-selectins, which is rapidly shifted on cell surface and the process is triggered by histamine and thrombin. E-selectin is entirely produced by endothelial cells on getting activated by various inflammatory factors such as TNF-alfa, IL-I and endotoxins [77]. All these three selectins can interact with sialylated, fucosylated or with sulfated glycans on proteoglycans, glycoproteins and glycolipids. The tetrasaccharide epitope Sialyl Lewis (a) and Lewis x have been found as a minimum ligands for binding with all three types of selectin. The validation of selectins as a target for anti-cancer therapy has been already realized in animal based models. A substantial decline in platelet-tumor cell thrombi formation has resulted in diminishing of metastasis has been reported in P-selectin deficient mice [78]. Role of E-selectin in metastasis has also been validated in transgenic mice with overexpression of E-selectin, which resulted in increase in liver metastasis [79]. Reduction in metastasis is also observed in two L-selectin knocked out mice models, thus actively suggesting the role of leukocytes in aggravating metastasis [80].

Heparin has the potential to inhibit selectins even with no similarity to selectin ligands [81]. The anti-coagulation effect of heparin increase time of cancer cells to be in blood vessels by limiting their adhesion to endothelium and platelets. This makes the tumor cells more vulnerable to get neutralized by NK cells in circulation [82]. Several laboratories have analyzed the effect of heparin on selectins. In one such experiment, the UFH is injected in P and L selectin-knocked out mice shortly after the injection of melanoma cells. [83-84]. Single bolus injections of heparin have been identified to reduce metastasis in wild type mice in a similar way as in P-selectin deficient mice [83]. In addition to this, a single heparin injection in P-selectin knock out mice produces no marked change on metastasis [74]. Interestingly, a single dose of heparin injected in L-selectin deficient mice just before to tumor cell injection resulted in further reduction in metastasis [80]. When heparin is injected shortly before or six hours after tumor cell injection, no further attenuation on metastasis in P- and L-selectin is observed. However, repeated injection of UFH causes increase in survival of mice, suggesting that heparin at such doses might have further antitmetastic activities apart from selectin blocker. A detailed characterization of non-anticoagulant heparin resulted in identification of specific heparin analogue (58% N-acetylated heparins), that holds outstanding P-selectin inhibition property while holding minimal heparinase activity and also with reduced growth factor interaction ability [85]. This heparin
analogue is found to reduce metastasis colon carcinomas and melanomas cells. Overall, these results strongly indicate that heparin block metastasis by interfering with selectin based cell-cell adhesion.

**Blocking of cell surface proteoglycans**

Another mechanism of heparin antimetastatic action may lies in its ability to compete with the HS group present on the Heparan sulfate proteoglycans (HSPGs) to bind with growth factors and its ability to compete with the HS group present on the Heparan sulfate. Blocking of cell surface proteoglycans may liberate these proteins from ECM [86]. Studies on human in vitro angiogenesis model proves that both UFH and LMWH are capable of inhibiting βFGF- induced angiogenesis by blocking the interaction of βFGF with HS. In man, therapeutic dose of UFH can indeed cause a hike in plasma levels of growth factors, such as βFGF [87-89]. Contradictory to this, Soker et al. demonstrate that LMWH but not UFH can block the interaction of growth factor to their high affinity receptors due to its smaller size [90-91]. Heparin fragment of less than 18 saccharide units has been identified to reduce the activity of VEGF [92] and fragment of less than 10 saccharide units can inhibit the activity of βFGF [93-94]. In one more such contradiction to UFH, the LMWH has also been demonstrated for inhibiting βFGF and VEGF-facilitated angiogenesis in vivo [95]. The true relevance of HSPGs and growth factor as a potential target for heparin is very perplexed and unpredictable.

**Heparin based nanoparticle and other innovative pharmaceutical approach to target tumors**

The major issue today’s modern chemotherapy is facing is harsh side effects of cytotoxic drugs due to their lack of specificity which can only be resolved by targeted drug delivery [94]. To overcome drug delivery issue, pharmaceutical modification in dosage form by making polymer drug conjugates are one of the approach that has been employed in past few years [90]. The approach has been shown to offer advantages in targeting tumor through a passive way by increasing the permeability and retention factor, which ultimately lead to reduction in noxious effect and increase in solubility as well as chemical stability. The advantage of polymer drug conjugates has been observed in a phase I trial where increase efficacy has been reported in forty-three patient with advanced solid tumor treated with cisplatin and poly (L-glutamic acid)-paclitaxel conjugate (PGA-PTX). Nevertheless, the challenges to find polymer with suitable physiochemical and biopharmaceutical properties are major hurdle in developing a selectively targeted polymer- drug conjugated therapy [95]. To deal with this issue, heparin, which is a biodegradable, non-cytotoxic, and water-soluble natural polysaccharide coupled with variety of pharmacological activities such anti-coagulation, anti-inflammation, anti-angiogenesis and anti-tumor [96], has allured intense attention. Taking advantage of this unique property of heparin, many heparin-drug conjugates have been developed for cancer chemotherapy [97]. In one such attempt, a LMWH based polymer drug conjugate holding two different anti-tumor drugs, Paclitaxel and all-trans-retinoic acid (ATRA) has been synthesized by Hou et al. The conjugate was reported to have less toxicity with better antitumor activity due to better permeability of nano-particles which increased the influx of paclitaxel (PTX) bound conjugate in tumor cells. The ATRA, which is known to induce regression of tumor by down regulating the survival factors of tumor and by activating of mitochondria dependent apoptosis, has played a significant role in the facilitating the transport of paclitaxel in the nucleus of tumor cell [98]. Beside heparin based nano-particles development, other approaches such as development of heparin based microcapsules loaded with anti-cancer drugs like doxorobucin has also take pace in past few years. In one such study doxorobucin has been encapsulated with layer-by-layer assembly of heparin and chitosan. Use of chitosan is mainly to prevent degradation of heparin from heparanase and also to facilitate the cellular uptake of heparin as the heparin is negatively charge that prevents it to move across the cell membrane, layer of positively charge chitosan applied to counter this effect. Thus, a synergistic response has been obtained against A549 cell lines [99]. Beside conjugation of two active anti-tumor agents, the attempt to make heparin-based nanoparticles also employed conjugation of florescent dye labeled heparin with gold nanoparticles.

Gold nanoparticles are widely used in many studies for their role in detection of biomolecules [100], selective marking of cells and proteins [101], killing tumor cells by hyper-thermal treatment and delivering therapeutic agent inside cells. In a study by Lee et al., heparin immobilized gold nanoparticles were prepared to optically identify the metastatic stage of tumor cells as well as to initiate apoptosis-induced tumor death by loading heparin into the cells. The nanoparticles were prepared by sealing the florescent heparin at the surface of gold nanoparticle via gold-thiol interaction to obtain florescence resonance energy transfer (FRET). These nanoparticles were further conjugated with arginine-glycine-aspartic acid, a cell adhesive peptide to make it more effective for inducing apoptosis. The study achieved acute apoptosis in β1,6 integrin expressing cancer cells due to targeted influx of heparin in cytoplasm of cancer cells [102]. In addition to all this innovations, Alam et al. recently conjugated a potent angiogenic analogue of heparin (LHT7) with tetrameric deoxycholic acid to prepare an oral formulation of heparin with better bioavailability [103]. The LHT7 has also been studied for its conjugation with reduced graphene oxide nanoparticles in a separate study. In this, light molecular weight heparin analogue coated with nano sheet of reduced graphene oxide is further loaded with doxorubicin. The particles are found to have a better dispersion in tumor vasculature of KB carcinoma bearing mice with better efficacy possibly due to synergistic effect of doxorubicin and LHT7 [104].

**Conclusion**

Heparin is the most negatively charged and important member of the GAG family and is commercially available in the market as a potential anticoagulant. Presence of heparin in various species has already been well documented but major commercial sources are still limited to porcine intestine and bovine lungs. Biological properties of heparin have been found to largely depend on its sulfation pattern. The properties also differ on the basis of its distribution in various vertebrates and invertebrates. The anticoagulant features of heparin have already been well studied and documented in various studies. The mechanism of anticoagulant activity of heparin is well established and known. Beside from its anticoagulant role, heparin has been explored in the past few decades for its anti-tumor properties, a role which gained much momentum after the development of some light molecular weight fractions of heparins (LMWH) along with unfractionated heparin (UFH) and non sulfated heparins with little or almost no anticoagulant properties. The process of tumor growth has been known to be highly complicated as many hallmarks of cancer such as angiogenesis and metastasis use different tactics to make the progression simplified for tumor cells. There is enough evidence suggesting heparin as a potential therapeutic agent against cancer. Various experimental studies have already demonstrated the efficacy of heparin in arresting tumor growth both in vitro and in vivo models. Clinical trials of some of the
LMWH have already shown better survival rate and regression of solid tumors especially in lung carcinoma patients. The clarity on its actual mechanism of action is still not clear. As some studies suggest that it interfere the progression of tumor by its anticoagulant property while other point out its interaction potential to engage cell adhesion molecules and proteoglycans. A synergistic and selective approach of heparin-based nanoparticles loaded with classical anti-cancer drugs such as paclitaxel and doxorubicin to target tumor cells, has been obtained by several studies. It can be concluded that heparin have a potential to serve itself as an anticancer agent or can complement the treatment in combination with other anti-cancer agent to minimize the harsh side effects and chemotherapy. Apart from the need to give much emphasis on a clear cut agent to minimize the harsh side effects and chemotherapy. These questions urgently needed to be answered so that design of prospective metastasis can take place.

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