

Acetylation may strengthen the antitumor activity of low molecular heparin

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Background: To synthesize acetylated low anticoagulant low molecular weight heparin (ALMWH) and to detect its antineoplastic activity.

Methods: We obtained Low anticoagulant low molecular weight heparin (LMWH) by splitting unfractionated heparin (UFH) with sodium periodate oxidation and sodium borohydride reduction, then the LMWH was subjected to acetylate catalyzed by dicyclohexylcarbodiimide and dimethylaminopyridine to produce ALMWH. The anti-proliferative activities were determined on MDA-MB-231 human breast cancer cells *in vitro*.

Results: ALMWH exhibited stranger anti-proliferative activity Compared with LMWH, In the MDA-MB-231 cell line, the growth of MDA-MB-231 cells with IC50 of 22.16 µM at 48 h in a concentrationdependent and time-dependent manner, ALMWH produced stronger inhibitory effects especially when it was used in low concentrations. By the use of bulky catalysts, the acetylation site in the molecular chain of low molecular weight heparin with a high selectivity, the synthesis process of Low anticoagulant low molecular weight heparin can be easily controlled. Therefore, large scale industrial production can be carried out.

Conclusions: The synthesized ALMWH possesses a high anti-proliferative activity, Chemical modification of structure can endow LMWH with a high antiproliferative activities. ALMWH is expected to enter clinical trials due to its high druggability. Simultaneously, this study provides a basic method for screening of antineoplastic drug with low toxicity.

Keywords: Acetylated low molecular weight heparin (ALMWH); MDA-MB-231 cells; antitumor; drug; heparan sulfate

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Introduction

Low molecular weight heparin (LMWH) has undergone sixty years of history since 1933. It has been used as an anticoagulant. Much research has shown that LWMH possesses antitumor activity except its anticoagulation and antithrombosis. The antitumor mechanisms may involve a double mechanism of the anticoagulation and non-anticoagulation. LMWH (1-7) promotes vascular

endothelium release tissue factor pathway inhibitor (TFPI), inhibits the highly expressed of tissue factor (TF) on tumor cells, suppresses local thrombin production on tumor and blocks the generation of local coagulation, reduces the formation of fibrin-platelet-tumor clot, and creates convenient conditions for the defense system to fight tumor cells. LWMH inhibits the activation of platelet, reduces the release of selectin, suppresses tumor cell adhere to the endothelial cell to limit adherence, invasion, and metastasis of tumor cell. The inhibition of LMWH on heparanase reduces the degradation of the enzyme on heparin sulfate (HS) and reduce the release of a growth factor such as basic fibroblast growth factor (bFGF) and Vascular endothelial growth factor (VEGF) (8-11), consequently suppresses the proliferation and blood vessel vascular formation of a tumor, and strengthen the barrier such as basement membrane and extracellular matrix blocking invasion and metastasis of tumor cells. However, like heparin, LMWH is limited in the antineoplastic application because of its low antitumor activity and insurmountable adverse reaction such as bleeding and thrombocytopenia. How to elevate LMWH's antitumor activity and lower its adverse reaction? A current research focus is forming.

In 2002, our group confirmed that heparan sulfate (HS) synthesized by normal human breast epithelial cells has a significant inhibitory effect on the proliferation of MDA-MB-231 and MCF-7 cells (12,13).

In 2008, Our group found that HS extracted from human breast cancer MCF-7 cells also has a significant inhibitory effect on breast cancer xenograft C3H mouse model (14), suggested that HS is an endogenous substance synthesized and secreted by cells and used to regulate their own proliferation activities probably. The low molecular weight heparin a glycosaminoglycan, is similar to heparan sulfate in structure and has anti-tumor cell invasion and metastasis activity (7). In 2015, it was found that acetylated Low anticoagulant low molecular heparin (ALMWH) was increased in alkalinity due to cationization, which changed from acidic oligosaccharides to amphoteric oligosaccharides. The affinity to the organism was enhanced and acetylation modification made this amphoteric oligosaccharide molecule. The chains are linear in aqueous solution and have a tendency to self-assemble into linear aggregates, which are easy to enter cells and enhance their anti-breast cancer cell proliferation activity (15).

Therefore, our group continues to research the antiproliferation effect and the mechanism on human breast cancer MDA-MB-231 cell.

Methods

We obtained LMWH and Unfractionated heparin (Jiangsu Wan Bang Company Limited, MW <8,000 Da), human breast cancer cell line (American Type Culture Collection), tributyl ammonium salt, acetic acid and acetic anhydride, DCC, DMAP (Sinopharm Chemical reagent Co, Lid), diethyl ether (The Wuxi City Chemical Co, Lth), CO₂ incubator (THERMO Forma), DMEM (Gibco), SCM (Hangzhou Four Seasons), and automatic cell counter (Shanghai RUIYU Biological Technology Co, LTD) for use in this study.

Preparation and characterization of the low anticoagulant acetylated low-molecular-weight heparin (ALMWH)

Low anticoagulant low molecular-weight heparin (LMWH) was prepared by the oxidation-reduction method. Briefly, heparin (1 g) was dissolved in water (10 mL), oxidized using sodium periodate (200 mg), and reduced using sodium borohydride (100 mg) to obtain LMWH (500 mg). LMWH (500 mg) was re-dissolved in water (10 mL). Thereafter, dimethyl sulfoxide (DMSO: 190 mL) was added to achieve a volume ratio of 5:95 for water to DMSO. The reaction was carried out at 50 °C for 2 h. The desulphated LMWH (300 mg) was obtained.

The desulphated LMWH (300 mg) was dissolved in distilled water (10 mL). Thereafter, Tri-n-ethylammonium (1 mL) was added to the eluent, lyophilized, and redissolved in dichloromethane. DCC (30 mg) and acetic anhydride (1 mL) were added to the solution with stirring for 4 h at 20 °C. Subsequently, NaOH solution (5%, 5 mL) was added dropwise to the reaction solution. After stirring for 7 h, the solution was filtered. The filtrate was adjusted to pH 7.0, decontaminated with diethyl ether, concentrated via decompression, and passed through the column. The desired fraction was collected and concentrated via decompression. The off-white powder of acetylated LMWH was obtained (ALMWH: 150 mg). The structures of LMWH and ALMWH were confirmed by ¹HNMR at 30 °C. The degree of acetylation was calculated according to the following formula:

Degree of acetylation (%) =

$$\left[\frac{\text{acetyl intensities (ALMWH) - acetyl intensities (LMWH)}}{\text{acetyl intensities (LMWH)}}\right] \times 100\%$$

[1]

Concentration of ALMWH and LMWH

The concentration of ALMWH and LMWH is prepared

according to the Weight concentration. The ALMWH and LMWH was dissolved in phosphate-buffered saline to prepare Different concentrations of ALMWH for use.

Effects of ALMWH on the growth of MDA-MB-231 cells

MDA-MB-231 cells were seeded at 2.5×10^4 cells/well into a 6-well tissue culture plate filled with 0.5mL of DMEM (containing 7% FBS) at 37 °C, 95% air, 5% CO₂, and damp-warm condition. Cells were allowed to grow for 48 h. Thereafter, the medium was changed to DMEM containing a low concentration of FBS (0.5%) and culture was continued for an additional 24, 48 and 72 h. After treatment with different concentrations of ALMWH, Free cells were released with trypsin, the cells were collected and the cell numbers were counted respectively, tumor growth curve was drawn and IC 50 value of tumor cells at were measured. The percent cell viability was calculated using the following formula:

Cell Viability (%) =
$$\left[\frac{\text{the cell number of intervening group}}{\text{the cell number of control group}}\right] \times 100\%$$
[2]

Statistical analysis

Statistical data are expressed as $\overline{x} \pm s$. Dunnett-*t* test was performed using SPSS 13.0 statistical software. Data are expressed as mean with 95% confidence intervals.

Results

Preparation of the low anticoagulant acetylated lowmolecular-weight heparin (ALMWH)

Using the redox method we have been successful degrade heparin into low anticoagulant low molecular weight heparin (LMWH), using bluk catalysts dicyclohexylcarbodiimide (DCC) and dimethylaminopyridine (DMAP), the The hydroxyl group of LMWH sugar chain was selectively acetylated, and the acetylated low anticoagulant low molecular weight heparin (ALMWH) was obtained. The acetylation rate of ALMWH was 12.0%, and the acetylation rate of low anticoagulation low molecular weight heparin (LMWH) was 3.32% by the analysis of proton nuclear magnetic of polysaccharide at 2 ppm.

ALMWH inhibits MDA-MB-231 cells growth in a dosedependent manner

ALMWH significantly inhibits MDA-MB-231 cell growth (*Table 1* and *Figure 1*). The inhibition tended to be enhanced with the increase of drug doses (*Figure 2*), and the antiproliferation of ALMWH on MDA-MB-231 cells is distinctly more significant than LMWH when the dose of ALMWH is equivalent to LMWH's dose.

ALMWH inhibits MDA-MB-231 cells growth in a timedependent manner

The counting results of the MDA-MB-231 cells after treatments of the different drugs, different doses of ALMWH, and different treatment times are shown in *Table 1* and *Figure 3*. The antiproliferative effects on MDA-MB-231 cells shows time-dependent when the dose of the given ALMWH is larger than 0.5 mg/mL. ALMWH's antiproliferation on MDA-MB-231 cells is beyond ALMWH, while the given dose of ALMWH is equal to LMWH (1.5 mg/mL). The growth inhibition of ALMWH treatment for 72 h on MDA-MB-231 cells is superior to the given dose of paclitaxel.

Cell viability study

ALMWH displayed dose-dependent cytotoxicity in the concentration range of 0.05-1.5 mg/mL. The IC50 of ALMWH in 48h on breast cancer MDA-MB-231 cells is 22.16 μ M.

Discussion

HS is synthesized and secreted by many cells; it is an endogenous antiproliferative molecule and exerts multifunction such as regulating cell adherence, proliferation, vascular generation, and tumor metastasis (16). Much research has proven that normal cell synthesized HS may inhibit the proliferation of tumor cells, suppress tumor vasculogenesis, and prevent tumor cell adherence, invasion as well as metastasis (14,17-21). The antiproliferative activity of HS exists in highly sulfated domains, which are enriched in hexuronate 2-sulfate residues and have a minimum length of 4–5 disaccharide units (22). The anticancer mechanism may involve inhibition of heparanase

Table	1 Shows the	a MDA-MB-2	231 cells cour	t treated by	different dru	gs, doses of A	LMWH an	d treating du	ation, $\overline{x} \times 10^{3}$	± SD, n=3				
		Ē		6 mc/ml)		E ma (m1)				ACRYL	LMWH			
Ч	ر	Ļ					0.05 (n	ng/mL)	0.15 (m	(JmL)	0.5 (m	ig/mL)	1.5 (m	g/mL)
	1×	SD	1×	SD	18	SD	1×	SD	18	SD	18	SD	١ĸ	SD
24	1.41	0.276	0.551	0.111	1.54	0.044	1.28	0.155	1.17	0.14	0.929	0.01	0.682	0.021
48	1.89	0.149	0.691	0.063	1.09	0.027	1.85	0.306	1.71	0.144	1.18	0.314	0.744	0.017
72	4.5	0.66	0.449	0.106	0.57	0.131	4.88	0.072	3.88	0.729	1.38	0.078	0.185	0.067
CTL, o	ontrol; PAC	 paclitaxel; 	ALMWH, aci	ryl low molec	cular heparir	 								

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Figure 1 Inhibition of ALMWH on MDA-MB-231 proliferation. (A,B,C) represent 24, 48, and 72 h of treating duration, respectively. CTL, control; PAC, paclitaxel; LMWH, low molecular heparin; ALMWH, acryl LMWH.



Figure 2 The antiproliferative effects of ALMWH on the MDA-MB-231 cells. ALMWH, acryl low molecular heparin.



Figure 3 Antiproliferative effects of different durations of drug treatment on MDA-MB-231 cells. CTL, control; PAC, paclitaxel; LMWH, low molecular heparin; ALMWH, acryl LMWH.

and on the expression of VEGF as well as induction of apoptosis. Thus, HS may be an excellent therapeutic candidate for cancer therapy. Unfortunately, the complexity of extraction and purification and low-yield rate for HS makes an outstanding contribution to limit its clinical application.

Like HS, HP is composed of the same repeating disaccharide subunits; however, its primary structure differs significantly from that of heparin. The disaccharide subunits are present in different proportions, with the GlcA/IdoA and GlcNAc/GlcNS ratios lower and the SO₄ content higher. The GlcNS to GlcNAc ratio is 1:1 in HS *vs.* >4:1 in heparin (23).

Heparin commonly used in the clinic is called unfractionated heparin (UFH). It is challenging to apply UFH to antineoplastic therapy though it can improve cancer patients' survival time (24-27), inhibit tumor growth and metastasis (27-34) because of its weak antitumor activity and more significant anticoagulant activity. LMWHs are the enzymatic or chemical degradation, and the mean molecular weight is about 5kD. The majority of LMWH chains are too short of forming LMWH-AT-IIa triple complex. Thus, LMWH has little anti-IIa activity compared to UFH (35). LMWH presents essential advantages compared to UFH: enhanced bioavailability after subcutaneous administration, prolonged half-life, and more interesting pharmacokinetic properties, reduced thrombocytopenia, and a more predictable dose-response (36-38). However, bleeding and thrombocytopenia adverse reactions still limit the larger dose and longterm use of LMWHs (39). Chain flexibility, the degree of sulfation (charge density), and the proper distribution of N-sulfate and N-acetyl groups (charge distribution) along the LMWH molecule provide convenience for chemical modification. Garg et al. found that O-sulfonation fully did not enhance the growth inhibition properties of UFH (40). Meanwhile, both N-sulfo and N-acetyl are essential for the growth inhibition properties (41), and the loss of 6-O-sulfo groups in GlcN residues decreases growth inhibition properties of UFH (42). Furthermore, the 2-O-sulfo group of L-iduronic acid residues in UFH are significantly crucial for its growth inhibition properties (43), and hexanovlation at 3-O-position increased the growth inhibition properties of UFH (44). Gohda et al. (45) found that the alkylated HP, after treatment with an oxidizing agent reduces the growth rate of SMCs by an increase in the alkyl group chain length. Earlier, Bârzu et al. (46) and Irimura et al. (47) also reported that growth inhibition properties of SMCs by O-acylated HP derivatives changed with the change in several carbon atoms present in their acyl chain. Other include N-acetylation, N-desulfation, O-desulfation, and carboxylreduction, and so on (48,49), chemical modification elevates the antitumor activity and reduce the anticoagulant activity as well as an adverse reaction of LMWH (50). We gained ALMWH through selective O-acetylation of LMWH, which shows excellent antineoplastic activity. The antineoplastic activity is stronger than the isodose LMWH, the growth inhibition of MDA-MB-231 treated for 72 h is slight the that of paclitaxel given dose. The result suggests that the O-acetylation of LMWH can elevate the antineoplastic activity of LMWH.

Conclusions

We designed and constructed an acetylated low anticoagulant low molecular heparin (ALMWH) with High biocompatibility and lower side effects. This system showed Enhanced antitumor effect to MDA-MB-231 cells *in vitro* and has potential benefit in cancer therapy.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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