

Antitumor Effect of Butanoylated Heparin with Low Anticoagulant Activity on Lung Cancer Growth in Mice and Rats

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Abstract: Whole unfractionated heparin can modestly decrease tumor growth, but the dose of heparin is limited by its anticoagulant properties. To overcome this limitation, we modified the chemical structure of heparin and have prepared a heparin derivative by *O*-acylating low molecular weight heparin with butyric anhydride, producing a more potent antiproliferative compound, which is only weakly anticoagulant so that the dose may be escalated without threat of hemorrhage. In this study, we investigated the effect of this chemically modified heparin, butanoylated heparin, on the growth of lung cancer *in vitro* and *in vivo*. We found that butanoylated heparin a) significantly inhibited lung cancer cell proliferation *in vitro* and lung cancer growth in mice and rats; b) had very low anticoagulant effect; c) had no significant toxicity on heart, liver, kidney and lung; d) significantly although modestly induced apoptosis and decreased expression of the cell proliferation pathway consisting of mutant p53, phospho-Rb and E2F1 expression in the tumor tissues. We also found that butanoylated heparin significantly inhibited CXCL12 and CXCR4 expression, suggesting that CXCL12/CXCR4 axis may be involved in regulation of tumor growth inhibition by heparin. We concluded that chemically modified butanoylated heparin has potent antiproliferative activity against lung cancer and may represent a new chemical therapeutic agent for cancer patients.

Keywords: Heparin, butanoylated, antitumor, lung cancer, mice, rats.

INTRODUCTION

Heparin, which has been used as an anticoagulant for more than 50 years [1], is a member of a class of acidic polysaccharides called glycosaminoglycans and consists of alternating residues of uronic acid and hexosamine covalently bound to serine residues of the serglycin core protein. Besides anticoagulation, heparin has a variety of other biological activities, such as regulation of lipid metabolism, control of cell attachment to various proteins in the extracellular matrix, binding with acid and basic fibroblast growth factors, and inhibition of arterial smooth muscle cell proliferation [2, 3]. In addition, studies have shown that heparin improves survival in cancer patients [4-6] and inhibits tumor growth and metastasis [7-15]. However, limited clinical use has occurred because of the relatively weak anticancer activity of whole heparin and the side effect of anticoagulation. Therefore, it would be desirable to find a heparin compound that possesses a high inhibitory activity on tumor growth with weak anticoagulant activity so that it could be used at high doses in human cancer victims.

Attempts to obtain heparin derivatives with high antiproliferative and low anticoagulant activity have been made before for cancer therapy [16, 17]. Lapiere *et al.* reported that a 2, 3-*O*-sulphated heparin, with diminished anticoagulant activity, significantly decreased the subcutaneous tumor growth of a human pancreatic adenocarcinoma in nude mice and prolonged the survival time of mice with lung metastasis of melanoma [16]. Yoshitomi *et al.* observed the same

effects as unfractionated heparin in anticancer cell growth *in vitro* and in the prevention of metastasis of Lewis lung carcinoma in mice by using a periodate-oxidized and borohydride-reduced heparin with low anticoagulant activity [17]. In addition, tumor inhibition by different low molecular weight heparins (LMWH) has also been studied [18-20]. However, the diversity of findings on heparin in experimental studies on cancer progression and metastasis suggest that further work is necessary because of the uncertain and variable effects of heparin [21, 22].

Our laboratory has had an interest in developing heparin or its derivatives as antiproliferative agents to treat the pulmonary vascular remodeling of pulmonary hypertension [23-28]. We found that a butanoylated low molecular weight heparin was a very strong antiproliferative agent for pulmonary artery smooth muscle cells (data not shown) and, as Barzu noted [29], was a very weakly anticoagulant compound. We hypothesized that this heparin derivative could inhibit growth of lung cancer *in vitro* and *in vivo* more effectively than unfractionated heparin.

MATERIALS AND METHODS

Chemicals

Upjohn heparin (batch #1209b) from beef lung, a gift from Pharmacia & Upjohn Inc., Kalamazoo, MI, was used as a control unfractionated heparin. Chemically modified butanoylated heparin, an *O*-butanoyl derivative of periodate-oxidized heparin fragments, was prepared by treating the tributylammonium salt of periodate oxidized heparin fragments with butanoic anhydride as described previously for hexanoylated heparin derivative by us [28]. Briefly, the tributylammonium salt (11.9 g) was dissolved in dry DMF

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(114 mL), was cooled to 0°C under an argon atmosphere. 4-Dimethylaminopyridine (0.695 g, 5.7 mmol), butanoic anhydride (26.2 mL, 0.1 mol), and tributylamine (27 mL, 0.1 mol) were successively added in single portion, and the reaction was allowed to proceed under argon at room temperature for 24 hours. After cooling to 0°C, 5 % NaHCO₃ in water (227 mL) was gradually added, and the solution was stirred at room temperature for 48 h. Excess NaHCO₃ was eliminated by slow, dropwise addition of 1 N HCl (~200 mL) until a pH of 4.0 was reached and then readjusted to pH 7.0 with 1 N NaOH (~150 mL). Cold denatured (95%) ethanol (5 L, 5 vol) was added with stirring. The sample was allowed to sit overnight at 4°C to afford precipitate. The precipitate was recovered by decanting and dissolved in 0.2 M NaCl (114 mL). The precipitation procedure was repeated by adding absolute ethanol (570 mL). The precipitate was recovered by centrifugation at 15000 rpm for 20 minutes, dissolved in water (114 mL), and passed through a column (300 mL) of Dowex50WX8(H⁺) cation-exchange resin and 600 ml was recovered. The acid was neutralized to pH 7.0 with 10 N NaOH and the solution was filtered through a 0.22 µm Millipore filter. After lyophilization, O-butanoyl heparin derivative (7.1 g) was obtained as an off-white powder.

Cancer Cell Lines

A549, a non-small cell lung carcinoma cell (NSCLC) line and DMS79, a small cell carcinoma cell (SCLC) line, were obtained from ATCC (Manassas, VA). The cells were grown in F-12K media with 10% FBS, streptomycin, penicillin and amphotericin B, for this study.

Cell Proliferation Assay

A549 cells were used for this *in vitro* proliferation assay. The cells were seeded at 0.83×10^4 cells/well in 6-well tissue culture plates, allowed to grow for 24 hours, and growth arrested for another 24 hours. The media was then changed to standard medium (with 10% FBS), growth arrest medium (with 0.1% FBS), or standard medium with heparins at different doses. After treatment with heparin or heparin derivative for 24 hours, cells were harvested for cell assays using a direct cell-counting proliferation assay. Percent growth was calculated as: [net cell growth in treated medium / net cell growth in standard medium] × 100, where the net cell growth = cell growth in standard or treated medium minus cell growth in growth arrest medium.

Animal Model

These animal experiments were approved by the Subcommittee on Research Animal Care at the Massachusetts General Hospital. Subcutaneous xenograft mouse as well as a rat model were used for this study. Strain-specific CB17SC-M severe combined immunodeficiency (SCID) male mice were obtained from Taconic Farmer Inc (Germantown, NY) and were 8 weeks old when used for this study. Mice were assigned to one of six groups randomly, 6-8 mice per group. Each mouse was inoculated subcutaneously on the flank with a single dose of 2×10^6 A549 or DMS79 cells in 50 µl of phosphate-buffered saline solution, cell viability > 95%. The NIH nude rats (Cr:NIH-rnu), weighing ~ 150 g, were obtained from the National Cancer

Institute at Frederick (NCI-Frederick, Frederick, MD). Each rat was injected subcutaneously with a single dose of 2×10^6 A549 cells on the flank.

Heparin Treatment

Butanoylated heparin was used for the animal studies. Upjohn heparin was used for control as a native unfractionated heparin. From the day of tumor cell injection, the animals were treated with heparin at designed doses subcutaneously daily for 14 days. Control animals were given saline subcutaneously daily.

Measurement of Tumor Growth

A tumor growth curve was used for evaluation of tumor growth during the period by measuring tumor volume. The tumor volume (in cubic millimeters) was measured at day 1, 4, 8, 12, 15, according to a formula $(L \times S^2) \times 0.5$ (L = long diameter of tumor, S = short diameter of tumor) [30].

Tumor Weight for Evaluation of Tumor Inhibition

At day 15 of tumor cell inoculation, the animals were sacrificed with 200mg/kg of pentobarbital and used immediately for determination of tumor weight and pathology as well as for biological analysis. Tumor weight was determined by weighing the wet tumor. Percent tumor inhibition was calculated according to the equation (tumor weight in control group – tumor weight in treatment group)/ tumor weight in control group × 100. Part of the tumor was fixed in 10% natural buffered formalin, embedded in paraffin, sectioned at 4-6 µm and stained with H & E for pathology evaluation. The rest of the tumor tissue was frozen immediately in liquid nitrogen for use in biological analysis.

Histology

The heart, liver, kidney and lung tissues from the mice were harvested, fixed in 10% natural buffered formalin and stained with H & E for histology analysis to evaluate toxicity of whole as well as butanoylated heparin.

Measurement of Coagulation Time

To demonstrate the anticoagulant activity of the chemically modified butanoylated heparin *in vivo*, the time to clot formation was used to measure coagulation time in glass tube manufactured by VWR International, West Chester, PA.

Apoptosis Assay

TUNEL assay was conducted by using TdT FragEL DNA Fragmentation Detection Kit (Oncogene Research Products, San Diego, CA), following the manufacturer's protocol. An apoptotic index was determined by a blinded investigator by counting the ratio of apoptotic cells to total cells in the tissue section.

Western Blot Analysis

The tumor tissue was homogenized in lysis buffer (1× PBS, 1% Igepal CA 630, 0.5% sodium deoxycholate, 0.1%

SDS with 10 mg/ml PMSF). Homogenized tissues were incubated on ice for 30 minutes in lysis buffer and then centrifuged at 14,000 rpm for 10 minutes at 4 °C. The supernatant was removed and saved. Supernatants were stored at -80 °C until analysis. The protein concentration of the lysate was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Protein samples were electrophoresed on SDS-polyacrylamide gel and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corporation, Bedford, MA). After an overnight incubation with PBS with 10% nonfat milk, the membrane was incubated with primary antibody for 1 hr at room temperature or overnight at 4°C. The membrane was washed with buffer and then incubated with a horseradish peroxidase linked secondary antibody for 1 hr at room temperature. After washing with buffer, the signals were detected using an enhanced chemiluminescence (ECL) Western blot detection -kit, Western Lightning (PerkinElmer Life Sciences, Boston, MA), and visualized by exposure to X-ray film. Quantification of protein expression was performed using NIH 1.61 image software.

Antibodies for Western blots included p27 (mouse monoclonal, clone 57, BD Biosciences Pharmingen, San Diego, CA), Rb (total Rb, mouse monoclonal, 4H1), phospho-Rb (p-Rb) (rabbit polyclonal, Ser 795) (Cell Signaling Technology, Beverly, MA), mutant p53 (mouse monoclonal, clone Pab 240) and E2F1 (rabbit polyclonal, clone C-20) from Santa Cruz Biotechnology, Santa Cruz, CA. GAPDH mouse monoclonal antibody (clone 6C5) was purchased from Research Diagnostics, Inc., Flanders, NJ. Goat-antirabbit IgG-HRP (sc-2004, dilution 1:3,000) and goat-antimouse IgG-HRP (sc-2005, dilution 1:3000) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from A549 tumor tissues using TRIzol RNA isolation reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Total RNA (2 µg)

was used to carry out RT-PCR to measure mRNA expression with Ready-To-Go Your-Prime First-Strand Beads (Amersham Biosciences UK Limited, Little Chalfont, Buckinghamshire, England) for reverse transcription and Platinum PCR SuperMix reagents (Invitrogen, Carlsbad, CA) for PCR according to the manufacturers' instruction respectively. The primer pairs for CXCL12 [31] and CXCR4 [32] and for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [33] were purchased from Sigma Genosys, Woodlands, TX. After RT-PCR, each sample was run in agarose gel (15.0 g/L) electrophoresis to ensure that the right-size product was amplified in the reaction. Bands were visualized using ethidium bromide and the gels were photographed under UV light. Quantification of RT-PCR products was performed using NIH 1.61 image software.

Immunohistochemical Staining for CXCR4

Anti-CXCR4 antibody (rabbit, polyclonal, 1:50) was obtained from AnaSpec, Inc., San Jose, CA. Immunohistochemical staining kit (Histostain-Plus kit, DAB, Broad Spectrum) was purchased from Invitrogen, Carlsbad, CA. The staining procedures were performed following the manufacturer's protocol.

Statistical Analysis

Statistics were performed using the computer program Statview (SAS Institute Inc., Cary, NC) with factorial ANOVA. All values were expressed as the mean ± SEM. Significance was set at p<0.05.

RESULTS

Butanoylation Enhanced Heparin Inhibition of Tumor Cell Growth *In Vitro*

Both Upjohn whole heparin and chemically modified butanoylated heparin significantly inhibited A549 lung cancer cell proliferation in a dose dependent manner, but the effect of butanoylated heparin was significantly stronger

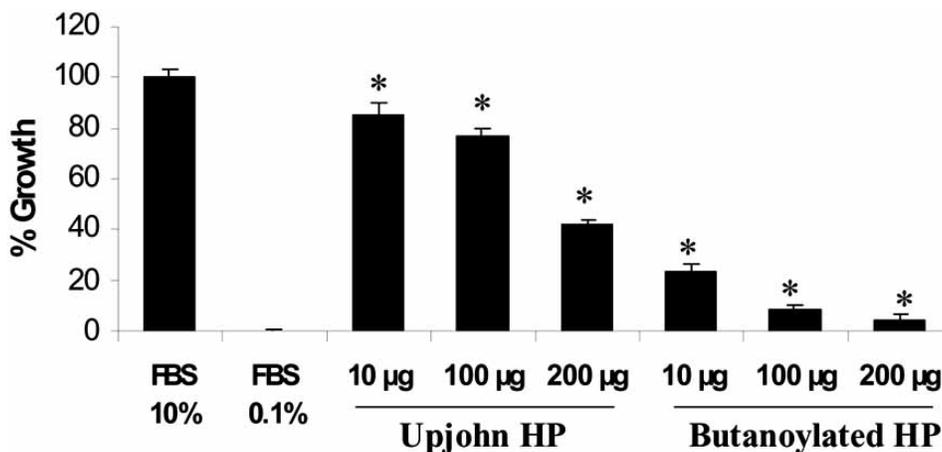


Fig. (1). Effect of chemically modified heparins on the growth of A549 lung cancer cells. A549 cells were incubated for 24 hours with Upjohn heparin (UHP) and butanoylated heparin (BHP) in the medium and then cell growth assay was performed. Cells grown in 10% FBS and 0.1% FBS were used for positive and negative controls. The results were representative of 3 separate experiments (total n =15) and error bars represent standard error. *p<0.05 as compared to 10% FBS and Upjohn heparin.

of butanoylated heparin was significantly stronger than Upjohn unfractionated heparin (Fig. (1)).

Butanoylated Heparin Inhibited A549 and DMS79 Tumor Growth in Mice

Butanoylated heparin significantly inhibited growth of tumors composed of A549 NCSLC cells (Fig. (2A)) or DMS79 SCLC cells (Fig. (2B)) in a dose dependent manner in the mice during 14 days of treatment. Although Upjohn heparin showed inhibition, most animals treated with high dose of Upjohn heparin died before two weeks and only one mouse at a dose of 100 mg/kg survived in both the A549 and the DMS79 tumor groups. All animals treated with butanoylated heparin survived.

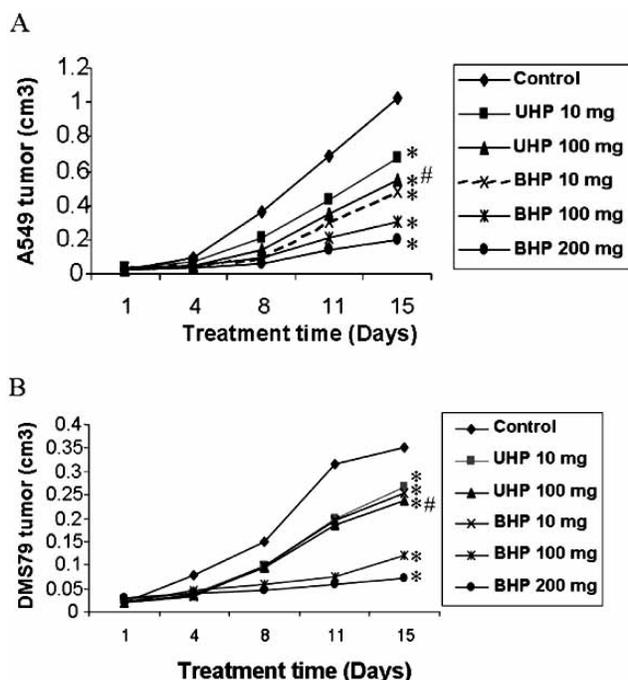


Fig. (2). Effect of chemically modified heparins on A549 and DMS79 tumor growth in mice. Growth curve for A549 tumor (A) and DMS79 tumor (B). * $p < 0.05$ as compared to control and regular Upjohn heparin. HP = heparin. $n = 6 - 8$ for each group except UHP 100 mg/kg ($\#n = 1$, other animals died before 14 days, although we initially used the same number of mice for each group).

Butanoylated Heparin *In Vivo* Increased Tumor Inhibition

Significant inhibition of tumor weight was observed in the mice treated with butanoylated heparin in a dose dependent manner in A549 and DMS79 tumor groups (Fig. (3A & B)). Although Upjohn heparin treatment decreased tumor weight, only one tumor was obtained from the mice at dose of 100 mg/kg, because all the other animals died before the end of the study due to bleeding. In the A549 tumor group, butanoylated heparin showed significantly stronger inhibition on tumor weight at a dose of 10mg/kg as compared to the same Upjohn heparin dose group (Fig. (3A)).

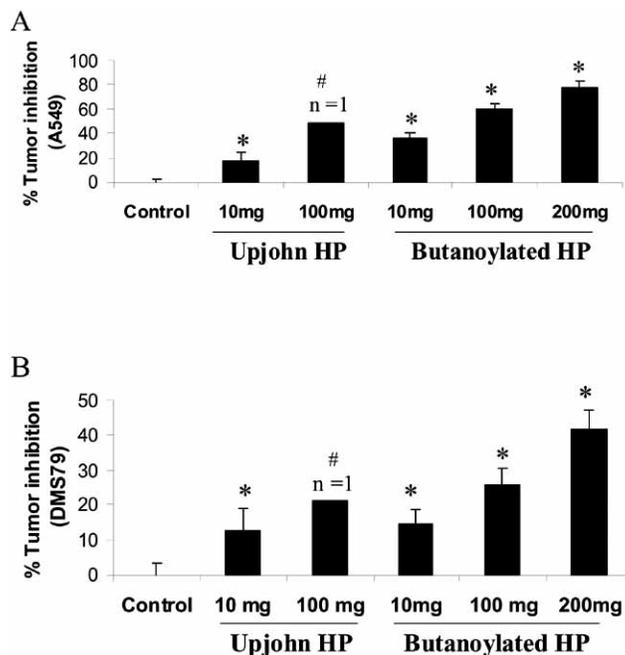


Fig. (3). Effect of chemically modified heparins on A549 and DMS79 tumor inhibition in mice. % Tumor inhibition for A549 (A) and DMS79 (B). * $p < 0.05$ as compared to control and regular Upjohn heparin. HP = heparin. $n = 6 - 8$ for each group except UHP 100 mg/kg ($\#n = 1$ other animals died before 14 days, although we initially used the same number of mice for each group).

Decreased Anticoagulant Activity of Butanoylated Heparin

Butanoylated heparin had a very low anticoagulant effect as compared with the regular Upjohn heparin. Significant intratumor bleeding was observed in the mice receiving Upjohn heparin at a dose of 100 mg/kg. However, no bleeding was observed in the tumor from the mice treated with butanoylated heparin at doses of 100 and 200 mg/kg (Fig. (4)).

No Significant Toxicity of Butanoylated Heparin on Mice

To determine the toxic effect of butanoylated heparin on mice at high doses, we examined the heart, liver, kidney and lung histology. The results showed no significant morphological change in the mice treated with different doses of butanoylated heparin and Upjohn heparin (Fig. (5)).

Butanoylated Heparin Induced Apoptosis

To investigate the mechanism by which heparin inhibited tumor growth, a TUNEL assay was performed to allow calculation of the percent apoptotic cell number. The immunohistochemical staining showed that this butanoylated heparin induced similar apoptosis to regular Upjohn heparin at 10mg/kg (Fig. (6)). However, 100 and 200 mg/kg of butanoylated heparin produced significantly more apoptosis than 10mg/kg of butanoylated heparin. Upjohn unfractionated heparin would not be used at higher doses because of hemorrhage (Fig. (4)).

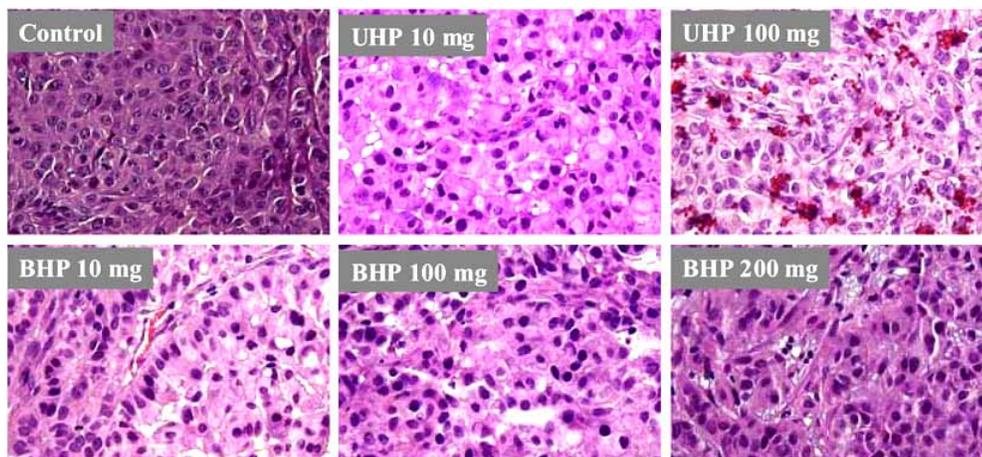


Fig. (4). Anticoagulation activity of butanoylated heparin. Tumor tissues grown in mice with or without heparin treatment were fixed with formaldehyde. The tissue sections were then stained with H & E for pathological evaluation. Representatives of tumor tissue histology showing significant bleeding inside the tumor tissue from mouse treated with 100mg/kg of UHP. No bleeding was seen in the tumors from other groups, even 100mg/kg and 200mg/kg of BHP. $n = 6 - 8$ for each group except UHP 100 mg/kg ($n = 1$). UHP = Upjohn heparin, BHP = Butanoylated heparin.

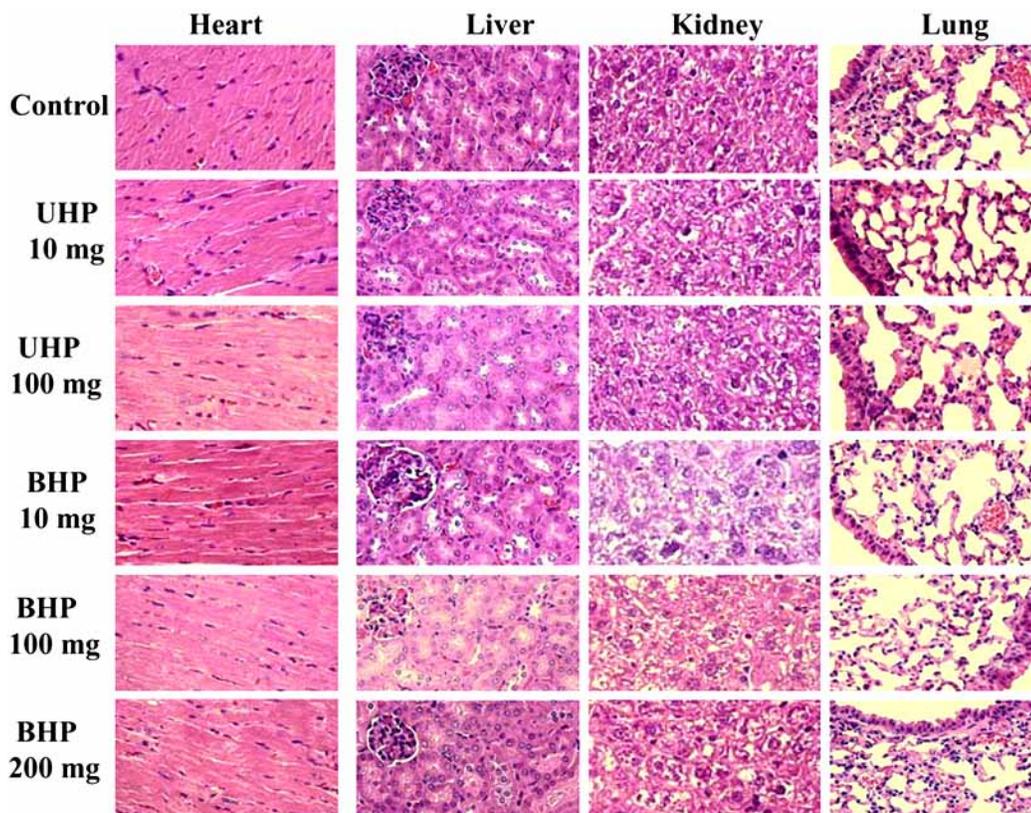


Fig. (5). Representative photomicrographs for histology from mice treated with or without Upjohn and chemically modified heparins. Tissues of the heart, liver, kidney and lung from mice with or without heparin treatment were fixed with formaldehyde. The tissue sections were then stained with H & E for pathological evaluation. Normal histological structures were seen in the tissues of the hearts, livers, kidneys and lungs. No significant change in pathology or bleeding was observed in the tissues from different groups. UHP = Upjohn heparin, BHP = Butanoylated heparin.

Butanoylated Heparin Decreased Mutant p53, Phosphor-Rb and E2F1 Expression

To determine the mechanism of action of heparin inhibition of lung cancer cells growth in mice, Western blot analy-

sis was used to analyze protein expression of the cell proliferation pathway consisting of p53, Rb and E2F from the implanted tumor tissues. The results showed that this butanoylated heparin significantly decreased mutant p53, phospho-

Rb and E2F1 expression in a dose dependent manner (Fig. (7A to D)).

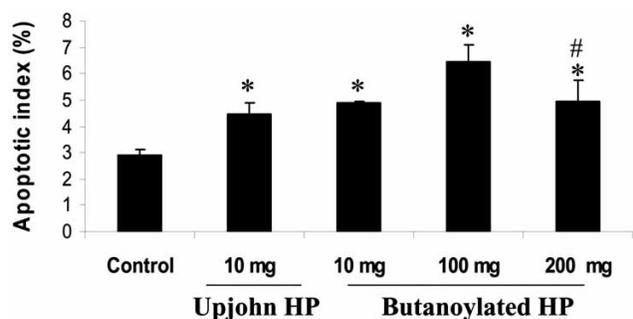


Fig. (6). Effect of butanoylated heparin on induction of cell apoptosis: TUNEL assay showing apoptotic index in the tumors from the mice treated with or without heparins. * $p < 0.05$ as compared to control and regular Upjohn heparin. The results were representative of 3 separate experiments. HP = heparin.

Butanoylated Heparin Regulated p27 Expression

Interestingly, overexpression of p27 protein was observed in the tumor tissue from control group by Western blot and immunohistochemistry. Butanoylated heparin significantly decreased p27 protein expression (Fig. (8A to C)).

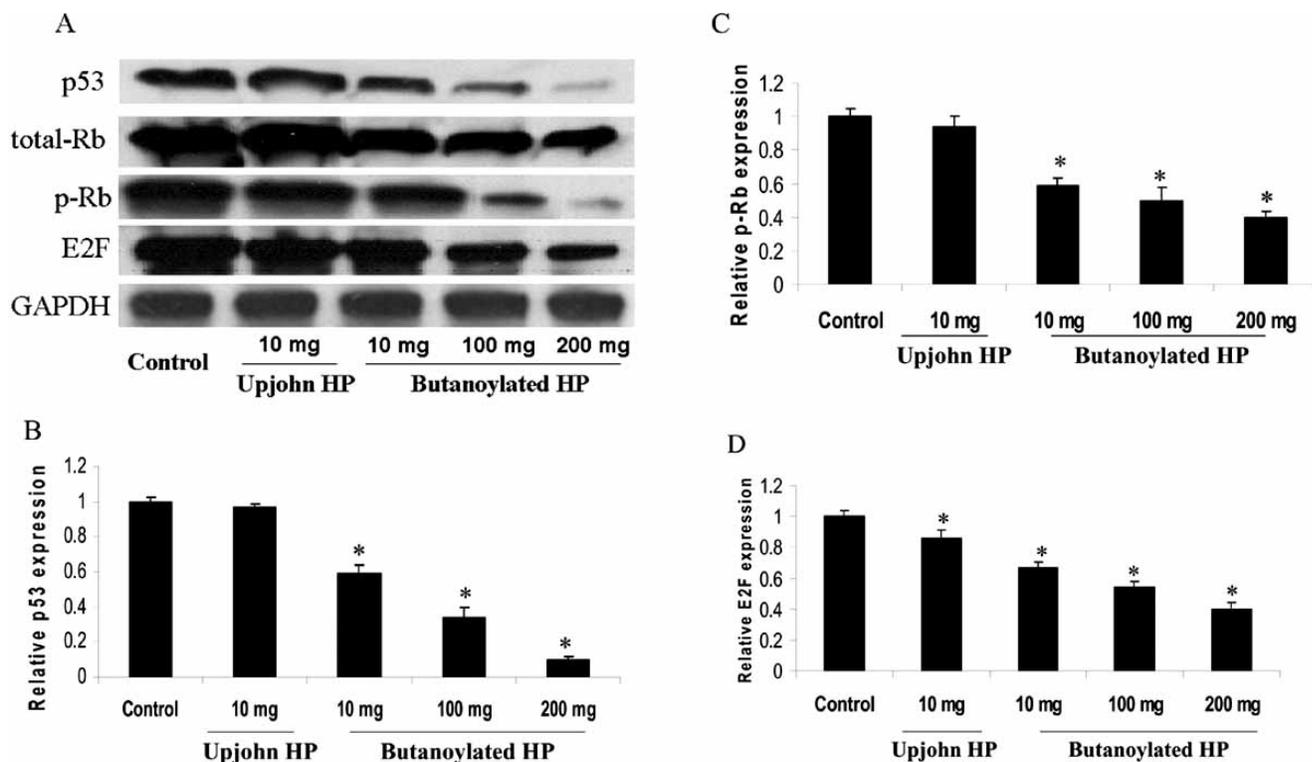


Fig. (7). Effects of butanoylated heparin on expression of mutant p53, Rb and E2F1: Total protein from the tissue was isolated and then subjected to Western blot analysis. GAPDH was used for equal loading control. The results were representative of 3 separate experiments. Representatives of Western blot for p53, total Rb and phosphor-Rb (p-Rb) and E2F1 (A). Quantitative analysis showing as relative protein expression for p53 (B), p-Rb (C) and E2F (D). * $p < 0.05$ as compared to control and regular Upjohn heparin. HP = heparin.

Butanoylated Heparin Inhibited A549 Lung Cancer Growth in Rats

To further confirm the results in larger animal, we used nude rats. We found that butanoylated heparin also significantly inhibited A549 tumor growth (Fig. (9A & B)) in nude rats at the doses of 40mg/kg and 100 mg/kg. There was a decrease, but not significant, in tumor growth and tumor weight at lower dose (Fig. (9A & B)). The loss of body weight was only observed at high dose (100 mg/kg) group from second week, but no significant change in body weight was observed in the animals treated with heparin at the other doses (Fig. (9C)).

Butanoylated Heparin Had Low Anticoagulation Activity in Rats

We measured clot time in rats treated with butanoylated heparin and regular Upjohn heparin respectively. We found that this chemically modified heparin did not prolong the clot time at doses of 40 mg/kg or less (Fig. (10A)). However, regular heparin significantly increased clot time at dose of 10mg/kg (Fig. (10B)).

Butanoylated Heparin Impact CXCL12/CXCR4 Pathway in Rats

To further investigate the mechanism by which heparin inhibits lung cancer growth in rats, we tested CXCL12 and

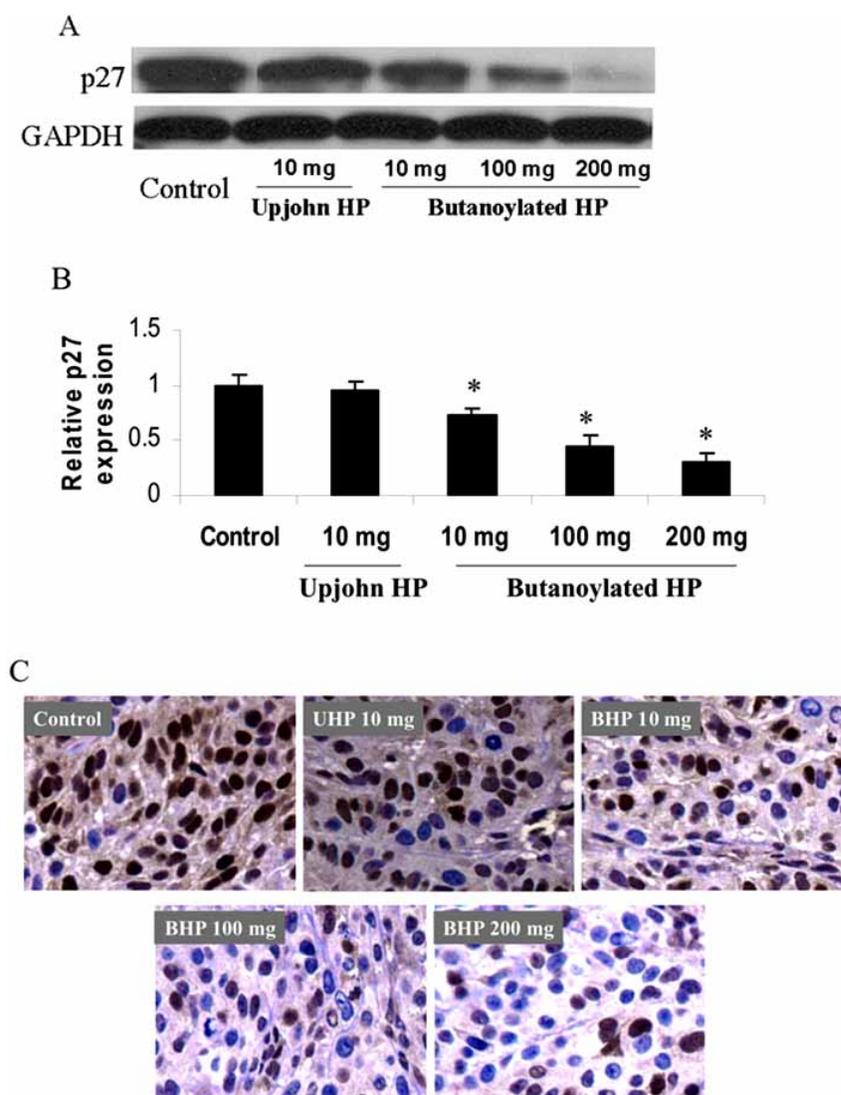


Fig. (8). Effects of butanoylated heparin on p27 expression: Western blot analysis showing relative p27 expression (A & B). Total protein from the tissue was isolated and then subjected to Western blot analysis. Representative Western blot (A) and quantitative data (B). GAPDH was used for equal loading control. (C) Representative microphotographs of immunohistochemical staining with p27 from tumor tissues. p27 positive staining cells (brown color) were seen in the tissues from all groups. There were more number of p27 positive cells in control slide and less in heparin treated animals. Fewer p27 positive cells and more p27 negative cells (blue color) were seen in butanoylated heparin mice. The results were representative of 3 separate experiments. * $p < 0.05$ as compared to control and regular Upjohn heparin. UHP = Upjohn heparin, BHP = Butanoylated heparin.

CXCR4 genes, because recent reports have shown that heparin binds to and inhibits CXCL12 and then impacts on the activity of its receptor CXCR4. We found that butanoylated heparin significantly inhibited expression of CXCL12 and CXCR4 mRNA (Fig. (11A)) and protein (Fig. (11B)) in the tumors from the rats treated with this heparin. Immunohistochemistry confirmed this in the tumor tissue grown in nude rats. (Fig. (12)).

DISCUSSION

The present study tested potential antitumor activity and the mechanism of action of butanoylated heparin, a whole heparin derivative, on lung cancer in a xenograft mouse and rat model. Our results showed that this heparin derivative had a more powerful effect on inhibition of lung cancer

growth (see Figs. 1, 2, 3, 9) with much less anticoagulant activity (see Figs. 4, 10) than regular heparin, and had no toxicity even at a very high dose (see Fig. 5). Our data also revealed that this heparin also inhibited tumor growth *via* inducing apoptosis, in association, with inhibition of the p53/Rb/E2F signaling pathway and CXCL12/CXCR4 (see Figs. 6, 7, 11, 12). Another interesting finding from this study was that p27, a cell cycle dependent kinase inhibitor, was overexpressed in lung cancer cells and heparin decreased its expression see Fig. 8). This is unlike the effect of heparin on vascular smooth muscle cells in which heparin increased p27 [34].

Antitumor effects of heparin have been reported, including antiangiogenesis [35-38], antitumor growth and metastasis [7-14, 18-20, 39-42], although one report showed that

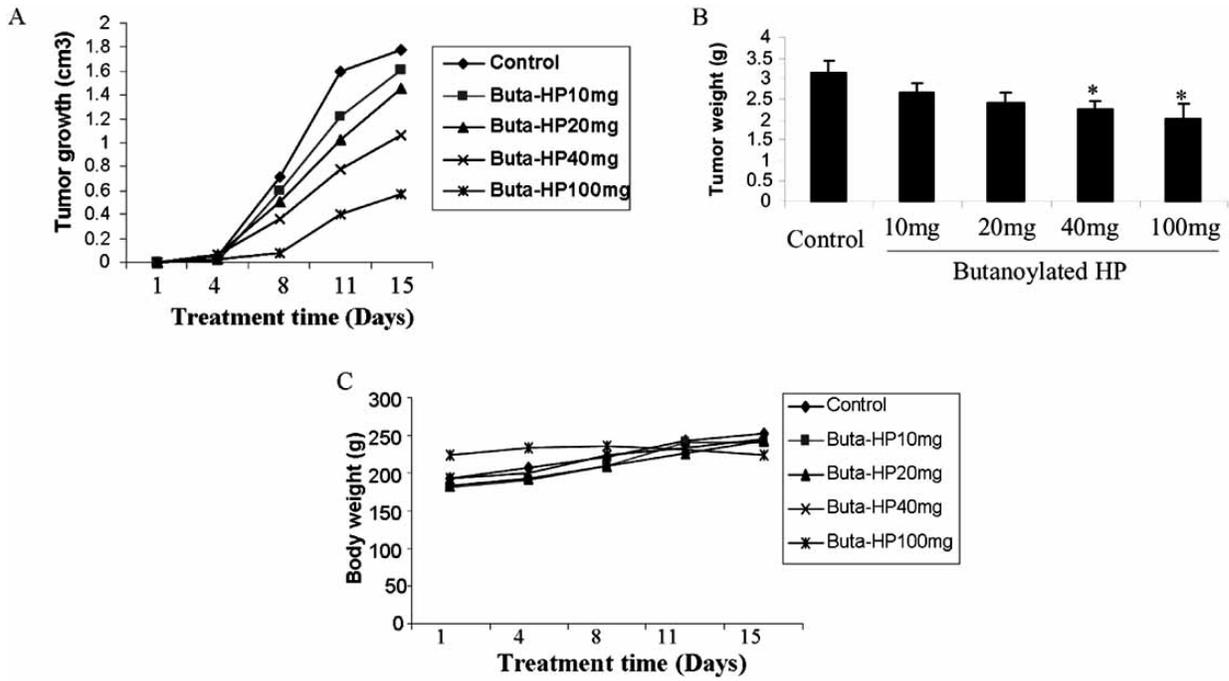


Fig. (9). Effect of butanoylated heparins on A549 tumor growth in rats. Growth curve (A) and tumor weight (B), and body weight (C). *p<0.05 as compared to control and regular heparin. HP = heparin. n = 5 for control and 3 for others.

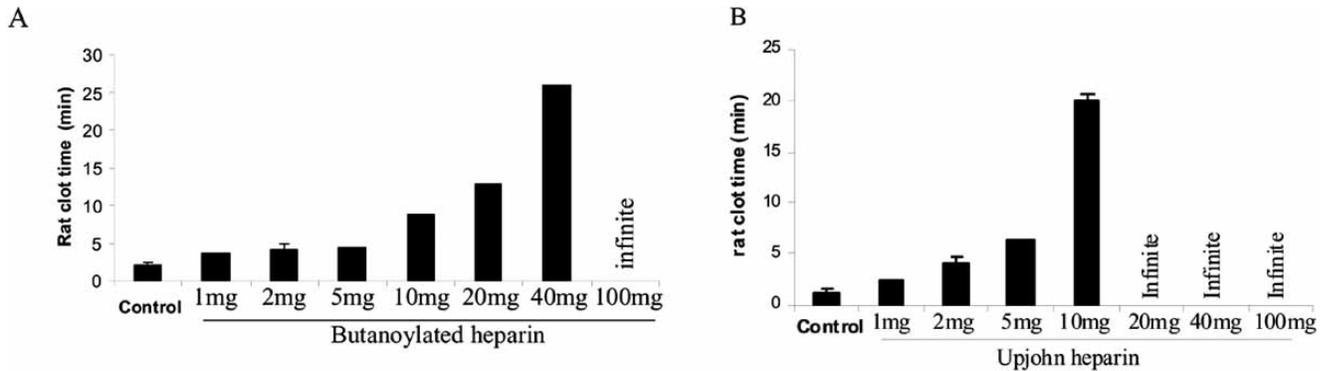


Fig. (10). Effect of butanoylated and regular heparins on clot time in rats. (A) showing coagulation time from rats treated with butanoylated heparin and (B) from rats treated with unfractionated Upjohn heparin. HP = heparin. n = 3 for each group.

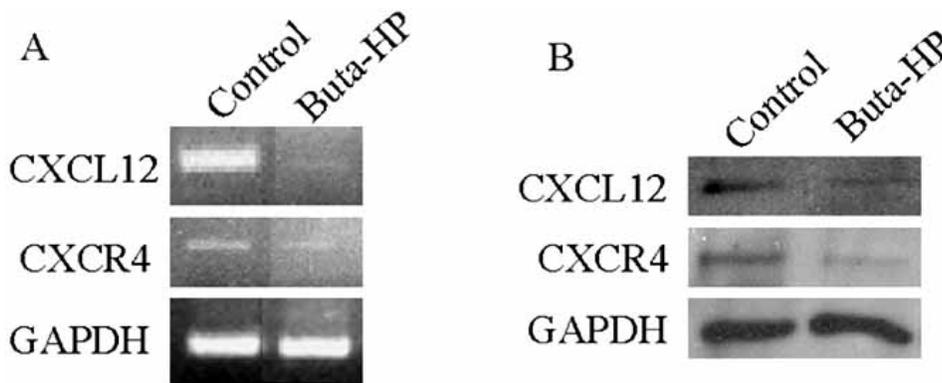


Fig. (11). Effect of butanoylated heparin on CXCL12/CXCR4 expression in tumors from rats: Total RNA and protein from the tissue were isolated and then subjected to RT-PCR and Western blot analysis. GAPDH was used for equal loading control. (A) CXCL12/CXCR4 mRNA and (B) CXCL12/CXCR4 protein. The results were representative of 3 separate experiments. Buta-HP = butanoylated heparin.

heparin increased human gastric carcinoma cell growth *in vitro* [43] and another study reported no significant inhibitory effect of heparin-deoxycholic acid nano-particles in a

mouse orthotopic lung cancer model [44]. In these reports, the heparins being used were unfractionated heparin and low molecular weight heparin (LMWH). Unfractionated heparin

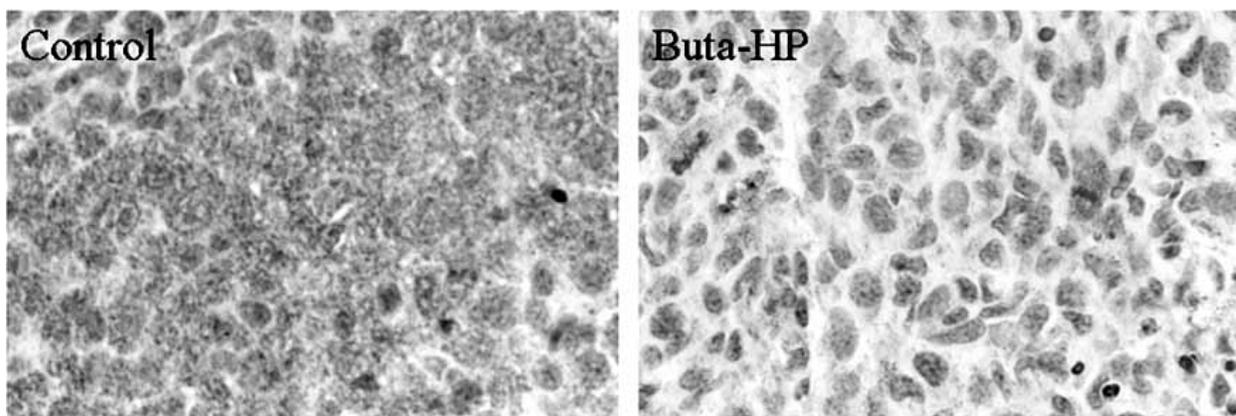


Fig. (12). CXCR4 immunohistochemical staining in A549 tumor tissues from nude rats: A diffuse and strong CXCR4 positive staining with brown color in cytoplasm was seen in the tissue from the control animals, but very weak positive staining in the tissue from the animals treated with butanoylated heparin. Buta-HP = butanoylated heparin.

has an antiproliferative effect, but also has strong anticoagulant activity, which limits its use in cancer treatment. Although LMWHs generally have less anticoagulant activity [16, 17, 29], the antitumor effect is not strong enough to be effective even at the larger doses allowed by the lesser anticoagulant effect. Yoshitomi *et al.* used a chemically modified heparin, periodate-oxidized and borohydride-reduced heparin with low anticoagulant activity (LAC heparin), to investigate antimetastatic activity in a Lewis lung cancer model. They found that, although both unfractionated heparin and LAC heparin inhibited the experimental lung metastasis, there was no significant difference between the two heparins [17]. Lapierre *et al.* showed their chemical modifications of heparin to have lower anticoagulant activity with still preserved antitumor and anti-metastatic properties, but they did not compare them with unfractionated heparin [16]. Bitan *et al.* reported inhibition by Fragmin, a LMWH heparin, on lung metastasis of melanoma in mice. They also did not show significant difference with fragmin compared with unfractionated heparin [45]. Barzu *et al.* reported that butanoylation of heparin increased by 15 fold the antiproliferative potency on cultured smooth muscle cells and showed a 4 fold lower anticoagulant activity [29]. However, they did not investigate the inhibitory effect of this heparin on tumor growth.

In this study, a chemically modified low molecular weight butanoylated heparin showed significantly stronger inhibition of lung cancer proliferation than regular heparin *in vitro*. This result indicated that the modification of heparin by butanoylation increased antiproliferative activity. To test *in vivo* antitumor activity of this new heparin derivative, we inoculated cancer cells from the cell lines A549 and DMS79 into the mice subcutaneously and then treated with heparin. We found that butanoylated heparin decreased the tumor growth of both A549 and DMS79 cells more effectively during two weeks treatment. Notably, all of the animals with butanoylated heparin treatment survived for two weeks, but most of the mice that received a high dose of regular heparin died before the end of the study due to bleeding. Also, significant bleeding into the tumor was observed from high dose regular heparin but no bleeding was seen in the high dose butanoylated heparin groups. This *in vivo* finding indi-

cated that this butanoylated heparin could be a potential therapeutic agent against lung cancer in humans because of its high antitumor activity and lower bleeding risk.

We found that heparins, neither Upjohn heparin nor butanoylated heparin, showed toxic morphologic damage to lung, liver, kidney and heart even though very high doses were given. The most common and fatal problem with heparin was anticoagulation that caused very severe bleeding at the sites of injection, but not toxicity to other organs. Although the bleeding started in the sites of injection, diffuse subcutaneous bleeding followed immediately, which resulted in the loss of blood volume, weakness and death of animals. If there were no bleeding caused by injection or other injuries resulting in tissue damage, the animals could survive; even though there was a significantly prolonged clotting time. In summary, we did not observe significant morphological changes in the heart, liver, kidney or lungs in the mice treated with different doses of unfractionated Upjohn heparin and chemically modified butanoylated heparin, including 100 mg/kg of UHP and 200mg/kg of BHP (see Fig. 5).

Although antiproliferative activity of heparin has been studied, the mechanism by which heparin inhibits cell proliferation has not been fully demonstrated. Tumor inhibition by heparin probably involves several different regulatory pathways [7], including suppression of protooncogenes, inhibition of heparinase activity, protection of TGF- β degradation and reduction of VEGF and bFGF activity. The action of heparin in inhibiting cancer cell proliferation also involves inhibition of P-selectin [9, 41, 46] and an increase in natural killer (NK) cell activity [47]. In this study, we found that butanoylated heparin significantly decreased mutant p53 expression and phosphorylation of Rb as well as expression of E2F. Because p53 plays an important role in regulating cell proliferation *via* the Rb/E2F pathway [48-51], mutation of p53 causes loss of its normal function and results in the accumulation or overexpression of non-functional p53, which is observed in most tumors. Downregulation of mutant p53 is associated with tumor growth inhibition and a better prognosis for cancer patients. Our finding suggests that heparin inhibited tumor growth in association with inhibition of cell proliferation regulating genes.

In this study, we observed that butanoylated heparin significantly increased the apoptotic index in tumor tissues. Previous reports suggested that part of the action of heparin was induction of apoptotic cell death [52-54]. Li *et al.* observed that unfractionated heparin induced apoptosis in a cultured nasopharyngeal carcinoma cell line [54]. Yu and colleagues reported that a chemically modified heparin induced apoptosis in tumor tissue [53]. Those observations indicated that induction of apoptosis was one of the mechanisms by which heparin inhibited cancer growth. We found that both 10mg/kg of Upjohn and butanoylated heparin (BHP) both significantly induced apoptosis in lung cancer cells compared to control. There was also a statistically significant increase in apoptotic index in BHP 10mg/kg group compared to UHP 10mg/kg group, although the difference was very small. We also found that 100 and 200 mg/kg of butanoylated heparin produced significantly more apoptosis than 10mg/kg of butanoylated heparin, although a diminished apoptotic index in the mice treated with 200mg/kg BHP was observed. Because a significantly greater effect at 200mg/kg butanoylated heparin was observed on proliferation markers than apoptosis (see Fig. 7), our data suggest that the effect of heparin as an antitumor agent was more on inhibition of cell proliferation than induction of apoptosis.

p27, a member of the cyclin-dependent kinase inhibitors, plays an important role in regulating cell proliferation and tumorigenesis [55, 56]. High expression of p27 is a favorable prognostic factor in cancer patients, including lung cancer [57]. Our previous studies have shown that heparin inhibition of PASM C proliferation was dependent on upregulation of p27 [34]. Horiuchi *et al.* reported that heparin inhibited leiomyomal smooth muscle cell proliferation accompanied by the induction of p27 [58]. Yatabe *et al.* previously also observed significantly increased p27 staining in human SCLC specimens [59]. In a later study from Yatabe's group, they suggested that high expression of p27 *in vivo* might favor the survival of SCLC by preventing apoptosis in a microenvironment unfavorable for cell proliferation [60]. However, in this study, we found that expression of p27 protein in xenografted lung tumor tissues was decreased by both Upjohn heparin and butanoylated heparin, although butanoylated heparin showed significantly more powerful inhibition of p27 expression. The mechanism by which heparin decreased the p27 in tumor tissue was not clear. Therefore, additional studies are necessary to determine the functional significance of p27 to heparin inhibition of cancer cell growth.

CXCL12, also known as stromal cell-derived factor-1 (SDF-1), is a chemokine. CXCR4 is the only receptor of CXCL12. CXCL12/CXCR4 axis is involved in a wide variety of physiological functions [61], including regulation of cell migration [61], tumor progression [62]. CXCL12/CXCR4 axis regulates cell function through complicated signaling pathways (www.proteinlounge.com/images/pathways). Studies have shown that CXCL12 can bind to heparin and then be negatively regulated by heparin [61, 63]. The inhibition of CXCL12 activity will affect its receptor, CXCR4. Recently, Mellor and co-workers reported that heparin and short-length oligosaccharides of heparin inhibited the lung metastasis of breast cancer cells and decreased CXCL12 and CXCR4 expression [64], which indicated that

CXCR4 was affected by heparin. Our present study found that butanoylated heparin significantly inhibited CXCL12 and CXCR4 expression. This finding suggests that our modified heparin may also inhibit tumor growth *via* the CXCL12/CXCR4 signaling pathway.

Mutation and inactivation of tumor suppressor p53 are observed in most tumors. Overexpression of CXCL12 and its receptor CXCR4 are observed in different cancers [63]. In this study, butanoylated heparin decreased mutant p53 expression and inhibited CXCL12/CXCR4. However, the relationship between p53 and CXCL12/CXCR4 in heparin inhibition of lung cancer growth is not clear. Recently, some interactions between them have been reported [65-70]. It was reported that wild type p53 repressed CXCR4 expression and that siRNA-mediated depletion of p53 increased CXCR4 expression in breast cancer cells [65]. Moskovits *et al.* found that p53 suppressed production of CXCL12 in cultured fibroblasts and suggested that p53 attenuated cancer progression through repression of CXCL12 expression in stromal fibroblasts [66]. Khan and co-workers' study observed that inhibition of CXCR4 activity decreased p53 and that CXCL12 increased p53 acetylation in neurons [68]. Khan *et al.*'s studies also observed the influence of CXCL12/CXCR4 on p53's downstream effectors Rb and E2F1. Their investigation indicated that CXCL12/CXCR4 were involved in cell cycle regulation, because they observed that CXCL12 increased Rb protein RNA levels and stimulated Rb activity in rat cortical neurons [69]. They also observed phosphorylation/activation of Rb and its effector E2F1 to be induced by CXCL12 [70]. However, a study from Schimanski and co-workers showed that p53 did not impact CXCR4, because they observed a high level of CXCR4 expression in a p53 dominant-negative transfect human hepatoma cell line [67]. These results from different laboratories suggest that further investigation is needed to determine the exact regulation between p53 and CXCL12/CXCR4.

The regulatory pathway that heparin, either unfractionated heparin or chemically modified heparin, uses to inhibit tumor progression has not been clearly delineated, although some investigations have been done. In this study and based on our previous work on pulmonary vascular smooth muscle cells [33, 34], we observed that both unfractionated Upjohn heparin and chemically modified butanoylated heparin can influence the expression of some genes, including p53, Rb, E2F and p27 and CXCL12/CXCR4. However, we could not conclude that these regulatory pathways are the determining signaling cascades that butanoylated heparin uses to inhibit cancer cell proliferation and tumor growth, because other mechanisms have also been reported, such as P-selectin and heparin inhibition of cancer [9, 17, 41]. Yoshitomi *et al.* [17] focused their study on the inhibitory effect of chemically modified periodate-oxidized and borohydride-reduced heparin on lung metastasis of lung carcinoma, but they did not show data on the mechanism. Gao and coworkers [9] showed their work on P-selectin and lung cancer cell adhesion *in vitro* by chemically modified heparins. They also did not report mechanistic data on which signaling pathway was used by the heparins. Besides Gao *et al.* other investigators also studied the relationship between P-selectin and cancer metastasis, for example that by Borsig and co-investigators [41]. However, they did not show data on selectin and inhibi-

tion of tumor growth. In addition, Chen and co-workers reported that chemically modified heparin derivate significantly inhibited L-selectin binding to human ovarian cancer cells [71]. Our work in this study was focused on antitumor growth, revealing that p53-Rb-E2F pathway was involved in this inhibition of lung cancer tumor growth.

In conclusion, this study has demonstrated that our chemically modified butanoylated heparin with much less anticoagulant activity than native heparin had a more powerful inhibitory effect on lung cancer growth than native heparin and no toxicity on heart, liver, kidney and lungs. Our data indicate that this novel heparin derivative may be useful as a new chemical therapeutic for cancer patients.

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ABBREVIATIONS

BHP	=	butanoylated heparin
LMWH	=	low molecular weight heparins
NSCLC	=	non-small cell lung carcinoma cell
SCID	=	severe combined immunodeficiency
SCLC	=	small cell carcinoma cell
UHP	=	Upjohn heparin

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