약학석사학위논문

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경구용 신생혈관억제제 LHbisD4와 항PD-1 항체의 복합투여에 의한 항암상승작용

2018년 2월

서울대학교 융합과학기술대학원
분자의학 및 바이오제약학과
박 용 환

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지도교수 변 영 로

이 논문을 약학석사 학위논문으로 제출함

2018년 **2**월

서울대학교 융합과학기술대학원

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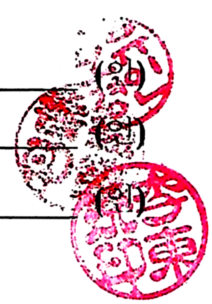
박용환의 석사학위논문을 인준함

2018년 **2**월

위 원 장 오 유 경 (인)

부 위 원 장 변 영 로 (인)

위 원 이 동 윤 (인)



Abstract

Angiogenesis, the formation of new blood vessels from pre-existing ones, is a crucial process in tumor growth, thus tumoral angiogenesis is a key contributor to aggressive tumor growth. In this study, we have developed a novel strategy for using a potent anti-angiogenic drug to not only inhibit tumoral angiogenesis, but also transform the tumor microenvironment into an immunosupportive state.

In this study we have successfully used LHbisD4, a previously developed heparin-based deoxycholic acid conjugate, as an orally active anti-angiogenic drug to induce potent anti-angiogenic and immunomodulatory effect *in vitro* and *in vivo.* By surface plasmon resonance (SPR) analysis we observed that LHbisD4 displays high binding affinity towards VEGF-A and treatment of LHbisD4 induced inhibition of VEGFR-2 phosphorylation in human umbilical vein endothelial cells (HUVECs), analyzed by western blot. LHbisD4 treatment in HUVECs also showed reduced proliferation by 82% compared to control and tubular formation was also reduced after treatment of LHbisD4. Their efficacy was successfully demonstrated in preclinical studies both as an anti-angiogenic and anti-tumor agent, and further its combination with αPD-1 antibody was evaluated to investigate the immunosupportive role of LHbisD4. The *in vivo* anti-tumor effect in mouse xenograft models showed 78.2% tumor growth inhibition compared to control. Through this study, we showed that LHbisD4 mediates a potent anti-angiogenic effect as well as an effective immunosupporting role, and potentiates the efficacy of immunotherapeutic agents in a synergistic manner.

Key words: Tumor angiogenesis, VEGF, regulatory T cell, immunotherapy, PD-1

Master's in Pharmacy

Student number: 2016-26009

Thesis advisor: Professor Young-ro Byun

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Figure 9. (a) Dose dependent inhibition of HUVEC sprouting by LHbisD4. (b) The number of sprouting in each group. (* p < 0.05; **p < 0.01; ***p < 0.0001; ns, not significant; student t test).

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Figure 12. Schematic diagram of combination

1. Introduction

1.1 Angiogenesis and tumor growth

Angiogenesis, which refers the formation of new blood vessels from pre-existing ones, is crucial process in tumor growth [1]. In healthy people, angiogenesis is an intricate multistep-process that takes place during embryonic development or wound healing. Angiogenesis is regulated by a balance between pro-angiogenic factors and anti-angiogenic factors. If this balance is disturbed in tumor microenvironment and pro-angiogenic factors become dominant over anti-angiogenic factors, it leads to excessive formation of new vasculatures. There are more than 30 known endogenous pro-angiogenic factors such as vascular endothelial growth factor (VEGF), angiopoietin and fibroblast growth factor (FGF) [2]. Tumor tissue requires nutrients and oxygen for growth and tumor angiogenesis supports aggressive tumor growth [3]. Moreover, tumor angiogenesis allows the migration of tumor cells into the circulatory system enabling the spread of cancer cells to other organs, and tumor metastasis follows [4]. This is the reason why anti-angiogenic inhibitors have risen as a promising therapy for various cancer types over the past decade. Pruning of tumor vessels by anti-angiogenic inhibitors such as AVASTIN (bevacizumb), a recombinant humanized monoclonal antibody for VEGF-A, has resulted in a remarkable increase in overall survival (OS) and progression-free survival (PFS) of patients suffering from metastatic cervical cancer, colorectal cancer and HER2 negative breast cancer.

Drugs	Main Targets	Indication
Bevacizumab (Avastin)	VEGF-A	Glioblastoma, Metastatic breast cancer, Metastatic renal cell carcinoma, Nonsquamous non small cell lung cancer
Afilbercept (Zaltrap)	VEGFR1, 2	Metastatic colorectal cancer
Sunitinib (Sutent)	PDGFR, VEGFR1, 2, 3, KIT, FLT3, CSF-1R, RET	Gastrointestinal stromal tumor after disease progression on or intolerance to imatinib
Sorafenib (Nexavar)	RAF, KIT, FLT-3, VEGFR1, 2, 3, RET, PDGFR	Unresectable hepatocellular carcinoma, Advanced renal cell carcinoma
Axitinib (Inlyta)	VEGFR1, 2, 3	Advanced renal cell carcinoma fatal failure of one prior systemic therapy
Vandetanib (Caprelsa)	EGFR, VEGFR, RET, BRK, TIE2, EPH receptor, Src	Treatment of symptomatic or progressive advanced medullary thyroid cancer
Cabozantinib (Cometriq)	RET, MET, VEGFR1, 2, 3, KIT, TRKB, FLT-3, AXL, TIE-2	Progressive, metastatic medullary thyroid cancer (MTC)
Regorafenib (Stivarga)	RET, VEGFR1, 2, 3, KIT, PDGFR, FGFR, TIE2, DDR2, TrkA, Eph2A, RAF-1, Abl	Metastatic colorectal cancer (CRC), Locally advanced, unresectable or metastatic gastrointerstinal stromal tumor (GIST)
Pazopanib (votrient)	VEGF1, 2, 3, PDGFR, FGFR, Kit, Itk, Lck, c-Fms	Advanced renal cell carcinoma, advanced soft tissue sarcoma

Table 1. FDA-approved anti-angiogenic drug list and main targets, indications

1.2 Vascular endothelial growth factor (VEGF) in angiogenesis

The vascular endothelial growth factor (VEGF) family is a typical pro-angiogenic factor and has several subtypes as VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placenta growth factor (PIGF) [5,6,7]. In various tumor microenvironments, VEGF isoforms show significantly higher levels of blood concentration than in normal conditions, and one of the reasons for this phenomenon is the harsh hypoxic conditions within the tumor tissue. It is well-known that oxygen concentration of the cell influences the regulation of gene expressions, including glucose transporters, erythropoietin and VEGF protein [8,9]. For this reason, VEGF is highly expressed in a various type of tumor cells. VEGF expressed from tumor cells is secreted and its stimulation of the receptor VEGFR on endothelial cells results in the development of new blood vessels which supply nutrients and oxygen to tumor cells for growth [10].

LHbisD4 is a heparin-based compound which was synthesized from low molecular weight heparin (LMWH) via conjugating with several dimer forms of deoxycholic acid (DOCA). With its property of specifically binding to VEGF-A, we strategize to inhibit the VEGF-A/VEGFR2 axis via orally active LHbisD4 (Table 2) [11].

Pharmacokinetic (PK) parameters of LMWH and LHbisD4 in rats

	C_{max} (µg/mL)	T_{max} (h)	AUC (µg*h/mL)	$t_{1/2}$ (h)	Cl (mL/h/kg)	Vd (L/kg)	MRT (h)	BA (%)
Intravenous administration								
LMWH (1 mg/kg)	- - -	- - -	8.4 ± 0.7	1.3 ± 0.2	0.09 ± 0.01	0.18 ± 0.02	- - -	- - -
LHbisD4 (5 mg/kg)	- - -	- - -	79.6 ± 15.7	2.0 ± 0.3	0.05 ± 0.0	0.14 ± 0.02	- - -	- - -
Oral administration								
LMWH (10 mg/kg)	0.7 ± 0.0	0.5 ± 0.0	2.5 ± 1.6	- -	- - -	- - -	4.4 ± 0.4	3.0 ± 0.3
LHbisD4 (10 mg/kg)	9.3 ± 1.4	3.0 ± 0.0	36.8 ± 7.2	- -	- - -	- - -	3.2 ± 0.6	23.4* ± 4.6

Results are the means ± standard error mean

C_{max}; Maximum effective concentration

T_{max}; Time to reach maximum effective concentration

AUC; Area under the concentration-time curve from 0 to 8 h

Cl; Clearance

Vd; Volume of distribution

MRT; Mean residence time

BA; Absolute bioavailability

* $p < 0.001$ vs LMWH

Al-Hilal, Taslim A., et al. JCI 126.4 (2016): 1251.

Table 2. Pharmacokinetic(PK) parameters of LHbisD4.

1.3 Relationship between VEGF-A signalling and regulatory T cell

Increased population of regulatory T cells (Treg) was detected in peripheral blood and tumor tissue of cancer patients. There are some possible mechanisms that explain this. The first reason is the immunosuppressive tumor microenvironment. A Treg expresses chemokine receptors such as CCR4, and CCL(chemokine ligand)22 produced from tumor cells can interact with Treg, mainly by CCR4. This chemokine gradient can lead the migration of Treg toward tumor tissue. Secondly, hypoxic condition also has a major influence. It is through the induction of CCL28 which promotes tumor tolerance and angiogenesis [12,13,14]. $CD25^{+}Foxp3^{+}CD4^{+}$ T cells (regulatory T cell) have key roles in suppressing immune functions in various type of tumors [15]. The expression level of foxp3 from Treg is relevant to expression of VEGFR2 on Treg. The level of foxp3 expression on Tregs correlaes to the level of VEGFR2; it is already reported that VEGFR2 modulates immunosuppressive function of Treg [16]. As an effective way to suppress Treg function, an anti VEGF-A approach can work effectively by blocking VEGF-A/VEGR2 axis (**Figure 1**).

Combination therapy of chemotherapy and bevacizumab showed reduced population of Treg rather than chemotherapy-treated group in tumor-bearing mice. Although in this report, there was no significant effect on Treg function, this strategy was effective in modulating Treg proliferation. Suppressing VEGF/VEGFR axis mostly resulted in reduced Treg population, but not the function of Treg [17].

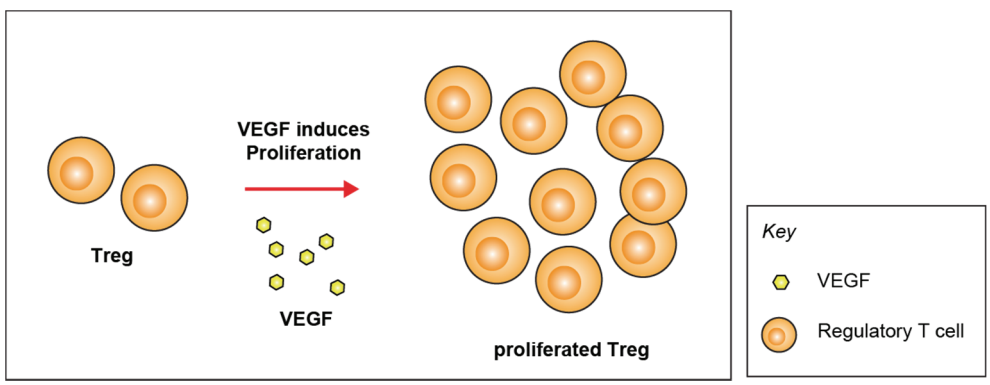


Figure 1. Scheme of relationship between VEGF-A and Treg proliferation

1.4 Cancer immunotherapy

Tumors have several mechanisms to avoid host immune responses for their survival. Co-inhibitory receptors such as Programmed Death-1 (PD-1), Cytotoxic T Lymphocyte-associated Antigen 4 (CTLA-4), B and T Lymphocyte Attenuator (BTLA) are known as immune checkpoint molecules playing crucial roles in regulating immune responses via different molecular ways [18, 19]. Various types of antibodies targeting these immune checkpoint inhibitor were developed and some of them were approved. Firstly approved drug, ipilimumab (anti-CTLA-4 mAb) successfully aimed at metastatic melanoma. Then, monoclonal antibodies targeting PD-1 or PD-L1 have been promising this past decade. PD-1/PD-L1 pathway is critical process in modulating immune reaction. PD-1 is a expressed on some immune cells such as T cells, B cells, natural killer cells, macrophages and dendritic cells [20]. The major function of PD-1 is to inhibit activation of T cells in peripheral tissues [21, 22, 23]. PD-L1 is upregulated on many cell types in response to proinflammatory cytokines, especially interferon-gamma [24]. Therefore, the co-expression of PD-1 and its ligand PD-L1 can modulate tissue destruction at inflammatory sites [25]. The increased expression of PD-L1 on many types of tumor inhibits anti-tumor immune response of T cell, and PD-1 expression on the tumor infiltrating lymphocytes (TILs) also reduces anti-tumor responses [26, 27, 28]. These two immune-related components are suggested as a potent pathway to block and enhance the anti-tumor immune response, using monoclonal antibodies [29, 30, 31].

1.5 Synergistic combination of LHbisD4 and αPD-1 antibody

As used clinically, many types of anti-angiogenic drugs such as bevacizumab (AVASTIN®), a humanized anti-VEGF monoclonal antibody, or sunitinib (SUTENT®), a small-molecule receptor tyrosine kinase inhibitor, have shown promised results in inhibiting tumor growth, and also its clinical limitation after long-term treatment. The most crucial reason was acquired resistance. Based on two different functions of VEGF-A, LHbisD4 effectively inhibits tumor angiogenesis and proliferation of regulatory T cell. Reduced population of Treg also has influence on the population of effector T cell. By combinating with αPD-1 Ab, anti-tumor immunotherapy will be synergistically enhanced.

2. Materials and Method

2.1 Materials, Cell lines

Low molecular weight heparin (LMWH) with an average molecular weight of 4500 Da was obtained from Nanjing King-Friend Biochemical Pharmaceutical Company Ltd. (Nanjing, China). Deoxycholic acid (DOCA), ethylchloroformate, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), hydroxysuccinimide (NHS), Methanol (MeOH), methylene chloride (MC), tetrahydrofuran (THF), 1,2-ethylene diamine (EDA), formamide (FA), dimethyl sulfoxide (DMSO), dimethylformamide (DMF), sodium cyanoborohydride, and 4-methyl morpholine (4-MMP), were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant human $VEGF_{165}$ (100-20-10UG) was purchased from PEPROTECH (Rocky Hill, NJ, USA).

Human umbilical vein endothelial cells (HUVECs) were purchased from PromoCell (Heidelberg, Germany). Murine melanoma (B16F10) were purchased from Korean Cell Line Bank (Seoul, South Korea). HUVECs were cultured in Endothelial Cell Growth Medium MV2 (ECGM MV2; PromoCell) supplemented with a SupplementMix solution and 1 % Antibiotic Antifungal agent, respectively. HUVECs were fasted in EBM Basal Medium phenol red free (CC-3129; Lonza).

B16F10 cells were cultured in high-glucose Dulbecco's modified eagle medium (Gibco, Carslbad, CA) supplemented with 10% fetal bovine serum (FP-0500-A, Atlas) and 1 % Antibiotic Antifungal agent(15240-062; Thermo Fisher Scientific), respectively. The cells were incubated in a humidified 5 % CO_2 atmosphere at 37 °C.

2.2 Synthesis and characterization of LHbisD4

2.2.1 Synthesis and characterization of bisDOCA

A dimer form of deoxycholic acid (bisDOCA) was synthesized by conjugating deoxcholic acids with L-lysine ethyl ester. Deoxycholic acid dissolved in tetrahydrofuran was purged with N_2 gas and reacted in an ice bath for 30 min, then 4-MMP and ethylchloroformate were added and reacted on room temperature for 4 hour. After the reaction was confirmed by TLC, L-lysine ethyl ester was added and refluxed overnight at 70 °C. The precipitation was eliminated by filtering and the solvent was evaporated. The residual compound was dissolved in chloroform and washed with 5 % HCl and 10 % NaOH and water successively. The organic layer was dried over Na_2SO_4, then filtered and evaporated. The concentrate was purified on a column packed with silica gel (0.04-0.06mm) and a mixture of 10 % MeOH/MC was used as a eluent. The major part was also purified to obtain compound. bisDOCA-ethyl ester was crystallized in ether.

A solution of bisDOCA-ethyl ester in ethanol was added dropwise with stirring up in EDA in an ice bath to form N-bisdeoxycholylethylamine (bisDOCA-NH_2). The mixture was purged with N_2 gas and left for 3 days in the dark. The solvent and excess EDA were evaporated in a vacuum condition and dissolved in a small amount of EtOH, then precipitated in cold water. The final product was purified by preparative adsorption chromatography on a column packed with silica gel (0.04-0.063 mm). The major part was purified in same method as described above. A mixture of chloroform, methanol and NH_4OH (ratio 7.75:3:0.25) was used as a eluent.

2.2.2 Synthesis and characterization of LHbisD4

The amine group of bisDOCA was coupled with hydrophilic backbone of LMWH. LMWH was dissolved in FA at 70 °C. bisDOCA was dissolved in a co-solvent system of DMF and FA. The carboxyl groups of LMWH were activated using EDC/NHS and reacted with bisDOCA at feed mole ratio of 1:12 in ice bath for 12 hr. The reacted compounds were precipitated in an excess amount of cold ethanol followed by freeze drying after centrifugation at 15,000 rpm at 4 °C for 30 min **(Figure 2)**. The synthesis of LHbisD4 was confirmed using ^{1}H NMR at 500 MHz. Sample was dissolved in D_2O and DMSO-d_6 as cosolvent (DMSO-d_6, 75%). The conjugation ratio of LMWH and bisDOCA was calculated by sulfuric acid degradation assay. In the sulfuric acid degradation assay, LHbisD4 and mixture of enoxaparin and bisDOCA were reacted with sulfuric acid in water at 70 °C. After heating, the absorbance of solution was measured at 420 nm by using UV/Vis spectrometer.

2.3 Binding affinity between LHbisD4 and VEGF-A by SPR

Biacore T100 (GE Healthcare, Sweden) was used for evaluating binding affinity between VEGF-A and LHbisD4. Recombinant human $VEGF_{165}$ was immobilized, which was adjusted to a level of 5,000 RU, on a sensor chip series S CM5 (GE Healthcare) by amide-coupling method. LHbisD4 and LMWH were prepared at concentrations ranging from 0.001 to 100 mM in HBS-EP plus buffer (GE Healthcare). HBS-EP plus buffer was used as a running buffer. The flow rate was set to 10 ml/min and 50 mM NaOH was used for regenerating surface of the

sensor chip after each cycle of analysis. The data were processed using the BIAevaluation software (GE Healthcare).

2.4 Inhibition of VEGFR2 phosphorylation by Western blot

After HUVECs has been cultured, culture medium was replaced to EBM for fasting cells and maintained for 24 hr. Then, $VEGF_{165}$ was treated (50 ng/ml) with or without LHbisD4 (10, 100 μg/ml), and incubated for 30 min. After 30 min, dishes were washed with cold PBS and remove PBS and add 1ml of cold RIPA buffer to 100mm cell culture dish. Adhered cells were scraped using the cell scraper on the ice. The cell suspension was transferred into a 1m Epi-tube and shaked for 30 min in 4 ℃. The samples were centrifuged at 16,000 g for 20 min in 4 ℃. Only the supernatant parts was retrieved and 10 μl of sample was taken and concentration was calculated by BCA protein assay. The concentration was adjusted using the Laemmli sample buffer and water and the lysate was boiled with sample buffer at 95 ℃ for 5 min. The sample was centrifuged at 16,000 g for 1 min. The equal amount of proteins was loaded for each sample into the SDS-PAGE gel (7.5% polyacrylamide gel) with SDS-PAGE molecular weight marker (Biomax 1000). The gel electrophoresis was conducted for 2 hour at 110V. After the gel electrophoresis, the gel was transferred onto a PVDF membrane using the transfer machine (Biorad Trans-Blot Turbo) at 30V in cold room overnight running. After transfer process is finished, the membrane was wased using Tris-buffered saline with 0.1 % Tween 20 (P7949 Sigma-Aldrich). After transfer, the membrane was blocked for 1 hour at room temperature with shaking in a 5 % BSA blocking buffer for 1 hour at 4 °C.

The blocking buffer was replaced to new one and each of antibodies (VEGFR2, phosphorylated VEGFR2, beta-actin) was added at 1:2000 ratio. Membranes were incubated for overnight at 4 °C with gentle shaking. Membranes were washed out using TBST for 3 times every 10 min. Anti-rabbit IgG secondary antibody (A0545; SigmaAldrich) with blocking buffer was added at 1:4000 and incubated in gentle shaking for 1 hour and then washed for further 2 hours. Western Blot detection kit (DG-WP100 EZ-western Lumi Pico) was applied to the membrane and membranes were placed onto OHP paper. The western blot imaging machine(LAS 4000; GE healthcare) was used to obtain the image.

2.5 HUVEC Proliferation assay

To evaluate the effect of LHbisD4 on HUVEC proliferation *in vitro*, CCK assay was performed. Briefly, HUVECs were plated onto a 96-well culture plate at a density of 5 x 10^3 cells per well in ECGM for 72 hr. Afterward, culture medium was replaced to EBM for fasting cells and maintained for 24 hr. After fasting cells, $VEGF_{165}$ was treated (50 ng/ml) with or without LHbisD4 (1, 10, 100 μg/ml), and incubated for 24 hr. 10 μl of CCK-8 solution was added to each well of plate and a plate was incubated for 4 hr. The absorbance of CCK-8 treated cells was observed under microplate reader at 450nm.

2.6 Tube formation assay using HUVEC

To determine the effect of LHbisD4 on HUVEC differentiation *in vitro*, we performed tube formation assay. Matrigel (BD Bioscience, San Jose, CA) was coated on a 96-well culture plate, and HUVECs were plated at a density of 5×10^4 cells per well in EBM. Human $VEGF_{165}$ was treated (50 ng/ml) with or without LHbisD4 (1, 10, 100 μg/ml), and incubated for 24 h. The tube formation was observed under inverted microscope (Eclipse TE2000-S; Nikon Instruments, Tokyo, Japan).

2.7 Spheroid sprouting assay using HUVEC

To assess the effect of LHbisD4 on angiogenic sprouting, we prepared HUVEC spheroids. HUVECs were suspended in 1:4 mixture of ECGM and methocel solution (1.2% w/v of methylcellulose in ECGM). HUVEC spheroids were prepared by incubating the cell suspension as hanging-drops at 37 °C for overnight in 100% humidity. The spheroids were collected and resuspended in the collagen matrix. The collagen matrix was added to 24-well culture plate and incubated at 37 °C for 1 hr to allow collagen to polymerize. Feeding medium containing 100 ng/ml of $VEGF_{165}$ was added to each well. For the treatment group, LHbisD4 was added to the feeding medium at a concentration of 1, 10, 100 μg/ml, each. After 24 hr incubation, spheroids were observed under inverted microscope (Eclipse TE2000-S; Nikon Instruments, Tokyo, Japan).

2.8 *In vivo* Experiment

All *in vivo* experiments using live animals were carried out in compliance with the relevant laws and institutional guidelines of Seoul National University.

Each of C57BL/6 mouse (Female, 6-weeks old, Orient Bio Inc. Korea) was injected subcutaneously with 1.0 x 10^6 B16F10 melanoma cells. All of mice were devided into 4 groups. When tumor volume reached 50mm^3, mice of LHbisD4 group were treated orally with LHbisD4 (10mg/kg) daily for 18 days. Mice of αPD-1 Ab group were treated intraperitoneally with αPD-1 Ab (10mg/kg) every 3days for 5 times. Mice of combination group were treated with both of LHbisD4 and αPD-1 Ab.

Tumor size was observed by measuring the minor and major axis of the tumors with electronical digital calipers. Tumor volume was calculated according to the following formula : (tumor volume) = (major axis) × $(\text{minor axis})^2$ × 0.52. Mice were euthanized after tumor volumes reached 2000 mm^3. Body weight of mice was monitored also.

2.9 Fluorescence activated cell sorter (FACS) Analysis

After all mice were sacrificed, tumor and spleen tissues were obtained and digested into single cells for Fluorescence activated cell sorter (FACS) analysis.

Tumor tissues were cut into very small pieces and mixed with enzyme solution (mixture of Dispase, DNase, Collagenase), then incubated for 30 min in 37 degree. Digested samples were filtered through 40 um strainer and centrifugated. After washing with media (DMEM supplemented with 2% FBS and 1% antibiotics solution), a portion was sampled for analysis of tumor infiltrating lymphocytes

(TILs) by staining with CD45.2 antibody (Biolegend 109828). Single cell suspensions of tissue samples were layered carefully on top of lymphocyte separation medium (LSM; MPBio 0850494) and centrifugated at 450g for 20mins at room temperature. Separated lymphocytes were collected and washed using media. After centrifugation, cells were resuspended in media and counted before analysis.

Spleen tissues were filtered through 40 um strainer and centrifugated. After discarding supernatant solution, RBC lysis buffer (Biolegend 420301) were treated and incubated for 5min on ice. After centrifugation, cells were resuspended in media and counted before analysis.

Splenocytes and lymphocyte separated from tumor cells were stained with CD45.2 antibody and divided into 2 different groups. One was double stained with CD8a antibody (Biolegend 100712) and CD3ε antibody (Biolegend 100306) for quantifying cytotoxic T cells. Other was double stained with foxp3 antibody (BD 563902) and CD4 antibody (Biolegend 100412) for quantifying regulatory T cells. FACS Aria Ⅱ(BD Bioscience) was used for analyzing both of fixed cells, and live cells

2.10 Statistical analysis

Data represents the means ± standard deviation. A one-way analysis of variance was used for the comparison of variables between groups. All set of data were considered as statistically significant when p-value was lower than 0.05

3. Results

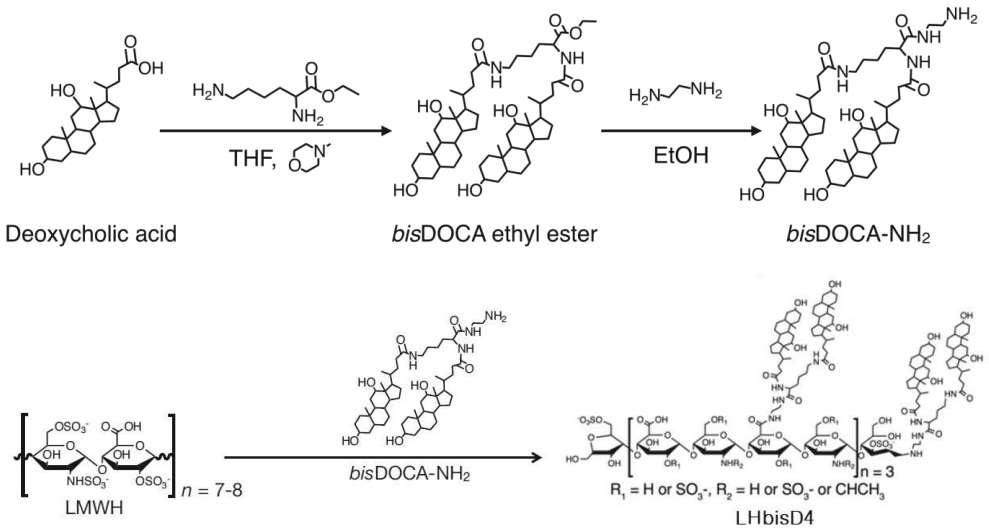
3.1 Characterization of LHbisD4

3.1.1 MALDI-TOF and NMR analysis

Synthesis of LHbisD4 was initiated from synthesizing bisDOCA **(Figure 2)**. In the synthesis of bisDOCA, each intermediate such as bisDOCA ethyl ester and bisDOCA-NH_2 were confirmed by MALDI-TOF **(Figure 3)**. The conjugation between LMWH and bisDOCA was confirmed by detection of the amide peak with ^{1}H-NMR which is presented at 3.0 - 5.0ppm **(Figure 4a)**. The peaks in the range of 0.8 – 1.5 ppm meant the presence of bisDOCA: (i) bisDOCA ethyl ester: MALDI-TOF (m/z): 923.58 [M], 929.62 [M+Li], 945.6 [M+Na], 962.59 [M+K]; calculated, 923.35. (ii) bisDOCA-NH_2: MALDI-TOF (m/z): 959.3 [M+Na]; calculated, 937.2.

3.1.2 Conjugation ratio of bisDOCA to LMWH

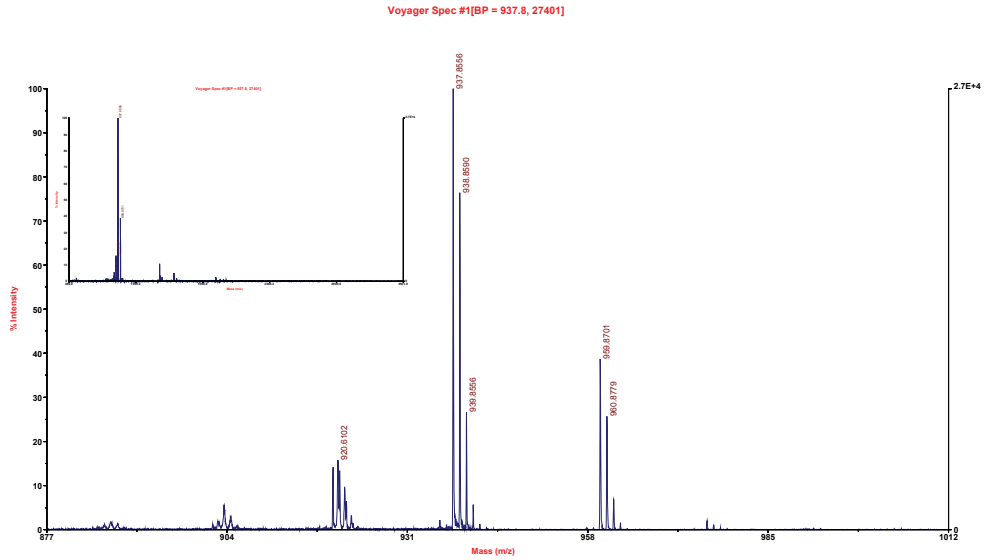
The conjugation ratio of bisDOCA to LMWH was determined by sulfuric acid degradation method, which is based on sulfuric acid's reactivity to the hydroxyl groups of either bile acid or LMWH; the product becomes chromogenic and shows absorbance on UV/Vis. Approximately 4 molecules of bisDOCA were conjugated to one molecule of LMWH **(Figure 4b)**.



Al-Hilal, Taslim A., et al. JCI 126.4 (2016): 1251.

Figure 2. Synthetic Scheme of bisDOCA and LHbisD4

(a)



(b)

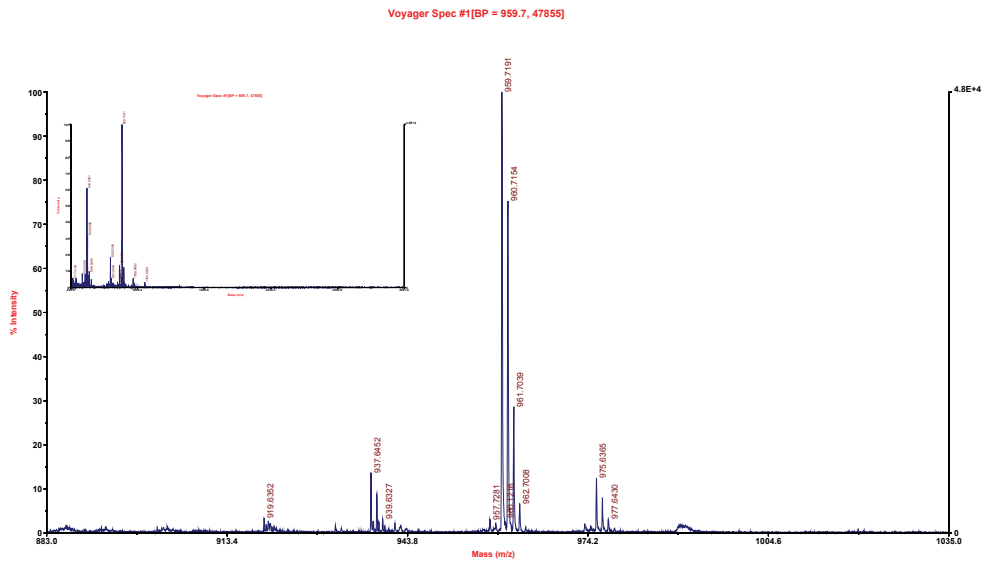
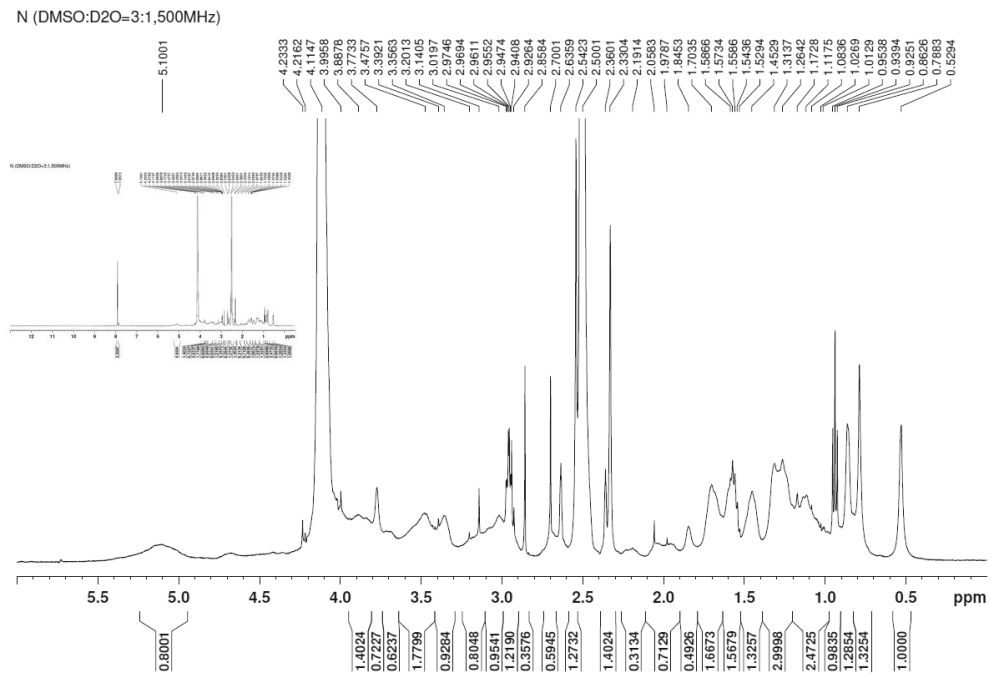


Figure 3. (a) MALDI-TOF spectrometry of bisDOCA-ethyl ester. (b) MALDI-TOF spectrometry of bisDOCA-NH_2

(a)



(b)

Method	Conjugation ratio
Sulfuric acid degradation assay	3.99

Figure 4. (a) ^{1}H-NMR result of LHbisD4. (b) Conjugation ratio of LMWH and bisDOCA calculated by sulfuric degradation assay

3.2 Anti VEGF-A agent, LHbisD4

3.2.1 SPR (Surface plasmon resonance)

The binding affinity of LHbisD4 and LMWH towards $VEGF_{165}$ was evaluated by SPR (Surface plasmon resonance). At first, the humanVEGF was immobilized on the surface of sensor chip by the amide-coupling method. LHbisD4 and enoxaparin were prepared at concentrations ranging from 0 to 100 μM in HBS-EP plus buffer. As a result, response units of LHbisD4 toward hVEGF was higher compared to LMWH **(Figure 5)**.

3.2.2 Western blot

For evaluating the degree of phosphorylated VEGFR-2, western blot was carried out. Phosphorylated VEGFR-2 was detected in HUVECs. (-) control group was fasted overnight, then collected. (+) control groups was treated with hVEGF after fasting overnight. Each of LHbisD4 group was treated with LHbisD4 with same condition as (+) control. From result of phosphorylated VEGFR-2, VEGFR-2 was not phosphorylated in (-) control because it was not treated with VEGF-A. (+) control showed band, which is diminished by treatment with LHbisD4 at a concentration of 10 μg/ml. On the other hand, bands referring VEGFR-2 was not significantly different in all groups **(Figure 6)**.

3.3 Anti-angiogenic effect of LHbisD4

3.3.1 Proliferation assay

HUVECs were cultured in 96-well culture plate. Control groups were treated with hVEGF after fasting overnight. Each of LHbisD4 group was treated with LHbisD4 with same conditions as control. After 24hr of treatment, CCK solution was added to all well and incubated for 3 hr. Proliferation of HUVEC was inhibited by treating LHbisD4 from concentration of 10 μg/ml. Proliferation of HUVEC was reduced by 21.5, 81.3 % respectively **(Figure 7).**

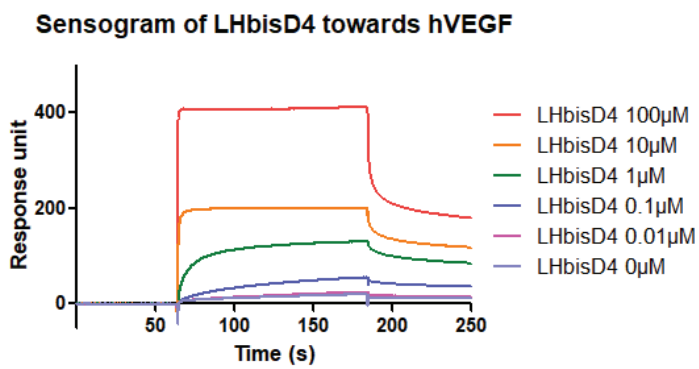
3.3.2 Tube formation assay

For demonstration of anti-angiogenic effect of LHbisD4, we performed tube formation assay which is an initial step in the angiogenic process. The degree of tube formation was evaluated by counting the number of nodes. The tube formation was stimulated by treating VEGF-A, but it was suppressed by treating additional LHbisD4. Anti-angiogenic effect of LHbisD4 was dose-dependent. LHbisD4 concentration of 10 μg/ml reduced the number of nodes by 44.3% compared to (+) control. LHbisD4 concentration of 100 μg/ml reduced the number of nodes by 91.9% compared to (+) control **(Figure 8)**.

3.3.3 Spheroid sprouting assay

The spheroid sprouting assay was performed to determine anti-angiogenic effect of LHbisD4. The HUVEC spheroids were used for evaluating angiogenic sprouting. The sprouting was induced by treating VEGF-A, but it was suppressed by treating additional LHbisD4. Anti-angiogenic effect of LHbisD4 was dose-dependent. LHbisD4 concentration of 10 μg/ml reduced the number of sprout by 40.4% compared to (+) control. LHbisD4 concentration of 100 μg/ml reduced the number of sprout by 44.4% compared to (+) control **(Figure 9).**

(a)



(b)

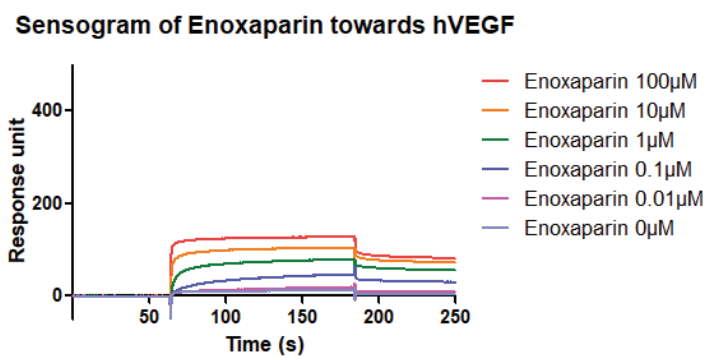
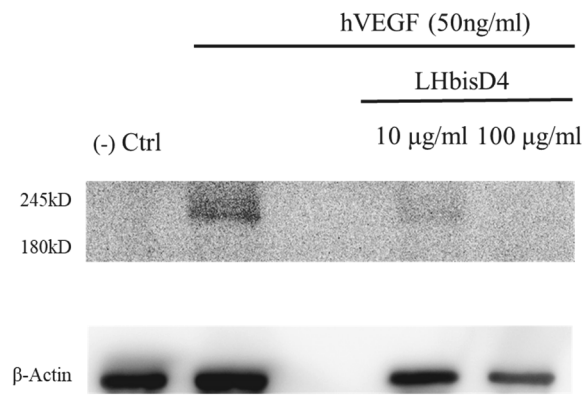


Figure 5. SPR Sensogram of (a) LHbisD4 and (b) LMWH towards human VEGF

(a) Phosphorylated VEGFR-2



(b) VEGFR-2

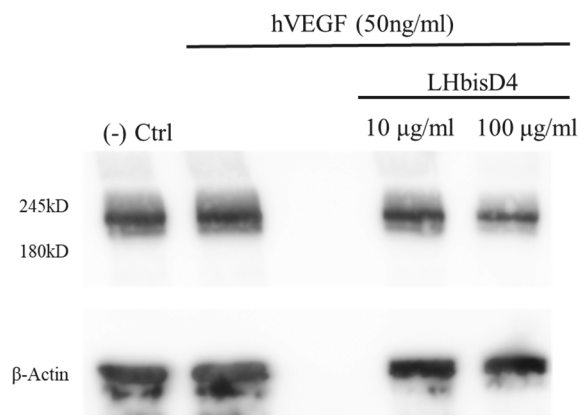


Figure 6. Western blot analysis. Phosphorylated VEGFR-2 was evaluated using HUVECs. (-) control group was fasted for overnight. (+) control groups was treated with hVEGF after fasting overnight. Each of LHbisD4 group was treated with LHbisD4 with same condition of (+) control. (a) Phosphorylated VEGFR-2. (b) VEGFR-2.

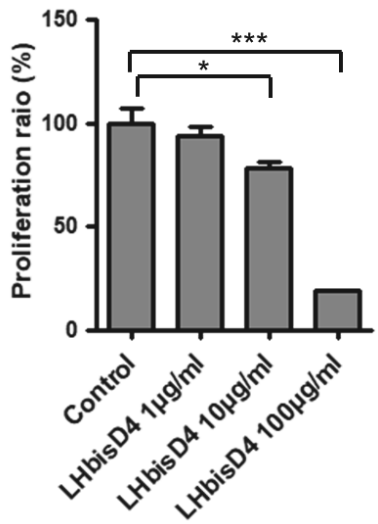
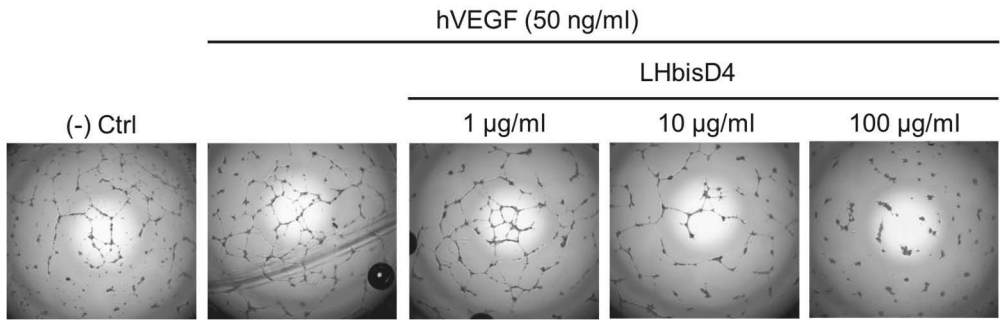


Figure 7. Proliferation assay using HUVECs. control groups was treated with hVEGF after fasting overnight. Each of LHbisD4 group was treated with LHbisD4 with same condition as control. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$; ns, not significant; student t test).

(a)



(b)

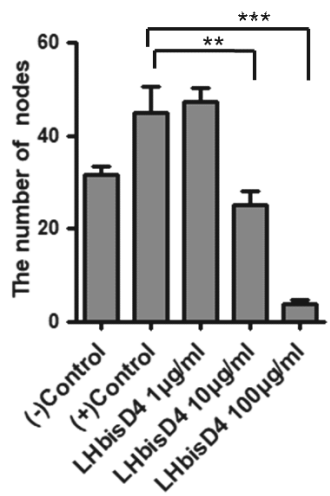
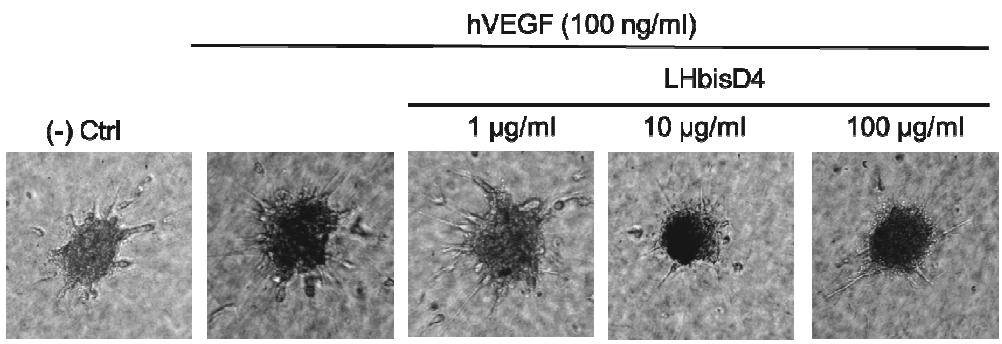


Figure 8. (a) Dose dependent inhibition of HUVEC tube formation by LHbisD4. (b) The number of nodes of each group. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$; ns, not significant; student t test).

(a)



(b)

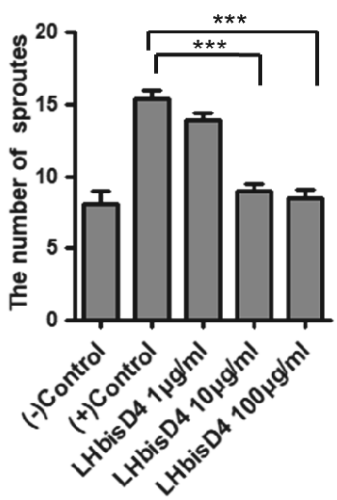


Figure 9. (a) Dose dependent inhibition of HUVEC sprouting by LHbisD4. (b) The number of sprouting in each group. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$; ns, not significant; student t test).

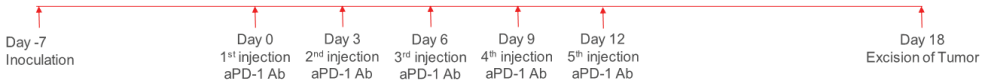
3.4 Tumor growth inhibition in *in vivo* mouse model

To evaluate anti-tumor efficacy of combination therapy, B16F10 melanoma cells were injected subcutaneously in C57BL/6 mice. All mice were divided into 4 groups. Treatment was initiated when tumor volume reached 50mm^3. For 18days of treatment, mice of LHbisD4 group were treated orally with LHbisD4 (10mg/kg) daily. αPD-1 Ab group were treated intraperitoneally with αPD-1 Ab (10mg/kg) every 3days for 5 times. Combination group were treated with both of LHbisD4 and αPD-1 Ab with same doses. Tumor growth was evaluated by measuring tumor volume. All mice were sacrificed and tumor tissues were harvested. Tumor volume of combination group was significantly reduced by 78.2% compared to control group. Tumor volume of LHbisD4 and αPD-1 Ab groups were reduced by 68.4%, 57.8% compared to the control group, respectively **(Figure 10b)**. And tumor weight of combination group was significantly reduced by 81.4% compared to the control group. Tumor weight of LHbisD4 and αPD-1 Ab groups were reduced by 58%, 55.4% compared to the control group, respectively **(Figure 10d).**

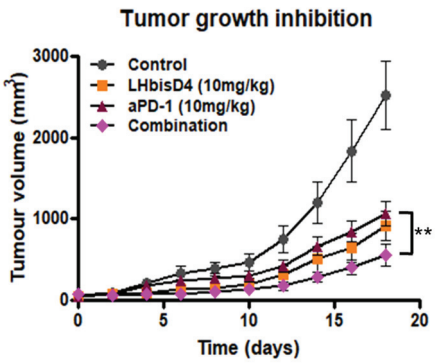
3.5 Fluorescence activated cell sorter (FACS) analysis

Fluorescence activated cell sorter (FACS) analysis was carried out for quantifying immune cells of different types. First of all, the proportion of whole lymphocytes was detected from tumor tissue. The amount of whole lymphocytes increased all drug-treated groups compared to control. Total lymphocyte in the combination group was higher by 97.5 % compared to control **(Figure 11c)**. Cytotoxic T cells (CTL) in tumor tissue increased by 171.1, 37.7 % for the combination treatment compared to control and αPD-1 Ab, respectively **(Figure 11d)**. Regulatory T cells (Treg) in the combination group did not show significant difference to control, however LHbisD4 group showed increased proportion of Treg by 37.4% compared to control **(Figure 11e)**. CTL in spleen in the combination group was increased by 41.3, 23.9 % compared to control and αPD-1 Ab, respectively **(Figure 11f)**. Overall, there was significant change between Treg amounts in spleen throughout all groups **(Figure 11g)**. In order to evaluate the functional aspects of the immune cell composition in the tumor and spleen tissue, ratio of CTL to Treg was calculated **(Fig 11.h-i)**. From tumor tissue, CTL/Treg of combination was increased by 146.6, 18.0 % compared to control and αPD-1 Ab, respectively. There was no significant difference between all groups from spleen tissue.

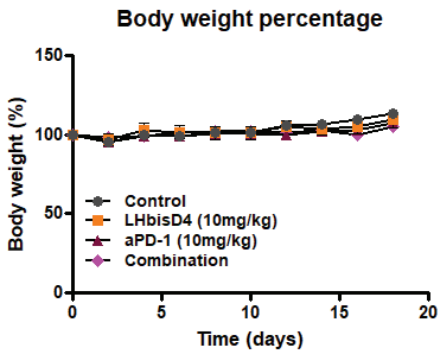
(a)


Day -7
Inoculation
Day 0
1st injection
aPD-1 Ab
Day 3
2nd injection
aPD-1 Ab
Day 6
3rd injection
aPD-1 Ab
Day 9
4th injection
aPD-1 Ab
Day 12
5th injection
aPD-1 Ab
Day 18
Excision of Tumor

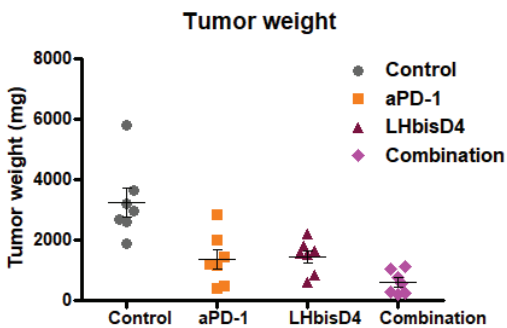
(b)


Tumor growth inhibition
Tumour volume (mm3)
Time (days)
Control
LHbisD4 (10mg/kg)
aPD-1 (10mg/kg)
Combination
**

(c)


Body weight percentage
Body weight (%)
Time (days)
Control
LHbisD4 (10mg/kg)
aPD-1 (10mg/kg)
Combination

(d)


Tumor weight
Tumor weight (mg)
Control
aPD-1
LHbisD4
Combination

(e)

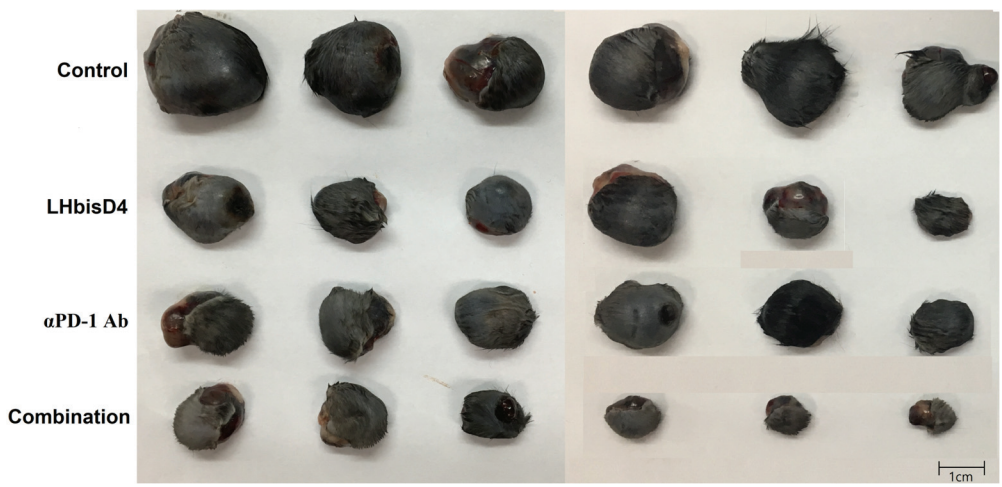
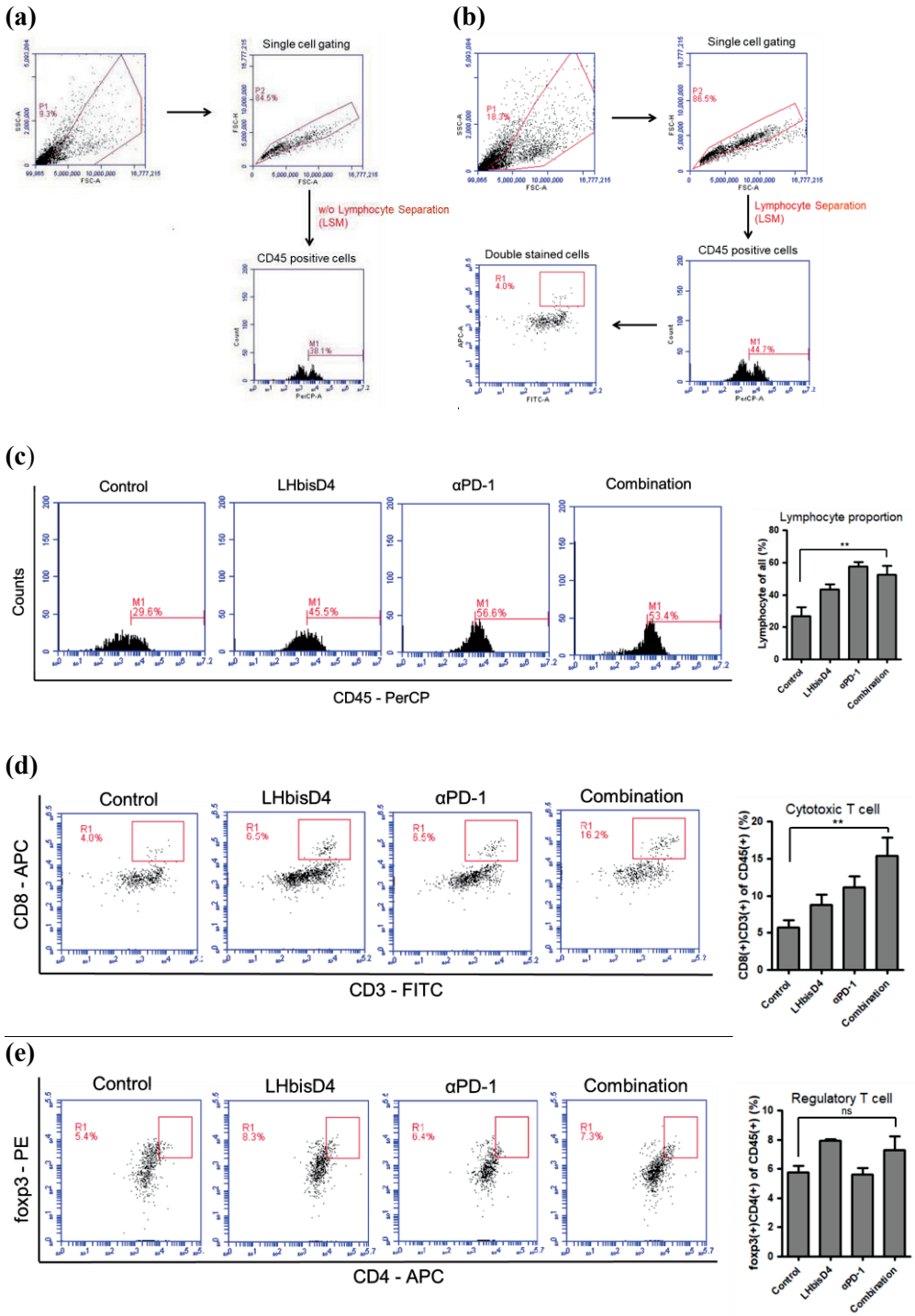


Figure 10. (a) Dosing schedule of *in vivo* experiment. (b) Tumor growth inhibition by treatment. (c) Body weight change percentage. (d) Tumor weight. (e) Representative image of tumor tissues after excision. Data presented as mean ± SEM (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$; ns, not significant; student t test).


(a)
Single cell gating
w/o Lymphocyte Separation (LSM)
CD45 positive cells
(b)
Single cell gating
Lymphocyte Separation (LSM)
CD45 positive cells
Double stained cells
(c)
Control
LHbisD4
αPD-1
Combination
Counts
CD45 - PerCP
Lymphocyte proportion
Lymphocyte of all (%)
(d)
CD8 - APC
CD3 - FITC
Cytotoxic T cell
CD8(+)CD3(+) of CD45(+) (%)
(e)
foxp3 - PE
CD4 - APC
Regulatory T cell
foxp3(+)CD4(+) of CD45(+) (%)
ns

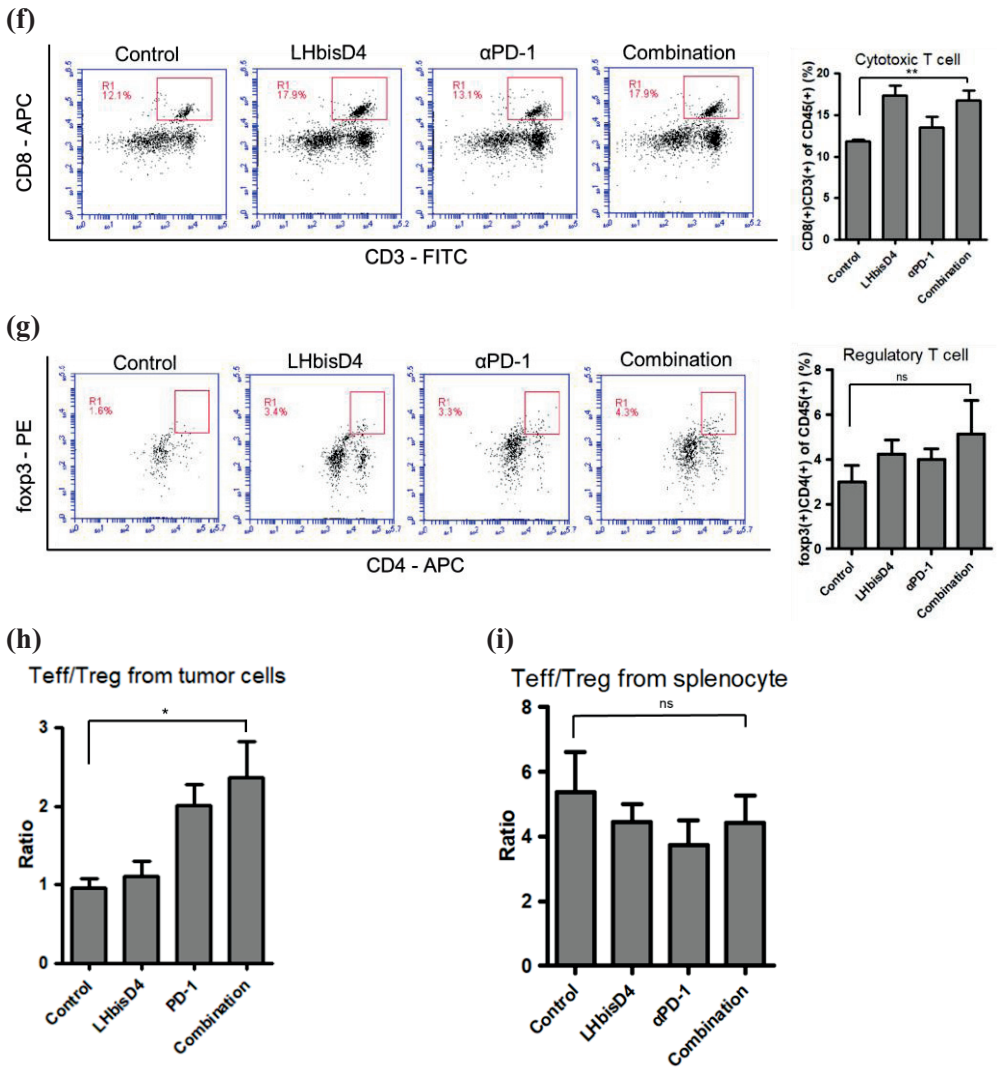


Figure 11. FACS analysis. (a) Gating strategy for lymphocytes stained for CD45, (b) from spleen and LSM(lymphocyte separation medium)-separated tumor tissue. (c) Whole lymphocytes from tumor cells stained with CD45 (d) Cytotoxic T cells from tumor tissue. (e) Regulatory T cells from tumor tissue. (f) Cytotoxic T cells from spleen tissue. (g) Regulatory T cells from spleen tissue. (h) Ratio of CTL to T_{reg} in tumor tissue. (i) Ratio of CTL to T_{reg} in spleen tissue. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$; ns, not significant; student t test)

4. Discussion

VEGF-A is well known as a pro-angiogenic factor and various types of angiogenesis inhibitors were developed for targeting VEGF-A. Discovering another role of VEGF-A, its immunosupportive function was focused in this study. By suppressing proliferation of Tregs, anti-VEGF-A therapy transforms the immunosuppressive tumoral microenvironment into immunosupportive one. Based on this, we hypothesized that anti-VEGF-A treatment enhances the efficacy of anti-tumor immunotherapy and to prove this hypothesis αPD-1 antibody therapy was evaluated for a synergistic combination.

In this study, the data shows that combination of LHbisD4 and αPD-1 antibody synergistically inhibits tumor growth. LHbisD4 blocks the VEGF-A/VEGFR-2 axis by suppressing VEGF-A; this results in not only inhibiting angiogenesis, but also enhancing the immune reaction.

LHbisD4, a heparin-based deoxycholic acid conjugate, was previously developed as an orally-active angiogenesis inhibitor. LHbisD4 showed higher binding affinity towards VEGF-A than LMWH. With this feature, LHbisD4-treated HUVECs showed less phosphorylated VEGFR-2 compared to control. This means LHbisD4 inhibits VEGF-A/VEGFR-2 signaling pathway, thus it can be regarded as an angiogenesis inhibitor and also play an immunosupportive role involved in proliferation of regulatory T cells. More *in vitro* experiments such as proliferation assay, tube formation and spheroid sprouting assay were carried out for demonstrating the anti-angiogenic effect of LHbisD4. Anti-tumor effect of LHbisD4 was observed when it was treated with concentration over 10 μg/ml, and

the effect was dose-dependent. From *in vivo* xenograft mouse model, combination therapy showed significantly boosted anti-tumor effect in B16F10-bearing mice. In FACS analysis, results were different from our hypothesis. The amount of total lymphocyte from tumor tissues was increased by treating with combination treatment. In the case of cytotoxic T cells and regulatory T cells, change in proportion for each immune cell was different in both of tumor and spleen. Cytotoxic cells increased by all drug treatments, and showed highest increase by combination treatment. For regulatory T cells, there was no significant difference between control and combination groups. In order to evaluate the functional aspects of the immune cell composition in the tumor and spleen tissue, ratio of CTL to Treg was calculated **(Fig 11.h-i)**. CTL/Treg ratio was increased by combination therapy from tumor tissue, but not from spleen tissue. This implies that combination treatment has an effect on the immune cells in tumor tissue.

Moreover, the proportion of regulatory T cells did not decrease in numbers by treating LHbisD4. Conversely, regulatory T cells slightly increased by treating LHbisD4 in both of tumor and spleen tissue. For this, we can consider the potential effects of vascular normalization and increased blood perfusion induced by LHbisD4 treatment. Low-dose treatment of angiogenesis inhibitor is well known for its vascular normalizing effect [32]. One of reason for increased regulatory T cell is considered as increased blood perfusion by vascular normalization. In order to clarify this, further analysis is needed such as immunohistochemical (IHC) analysis for quantifying tumor vasculature or doppler method for monitoring blood flow. Furthermore, considering the low expression of foxp3 from B16F10 melanoma,

other foxp3-rich cell lines such as CT26 colon cancer or 4T1 breast cancer could be more suitable for further in *vivo* experiments in this study.

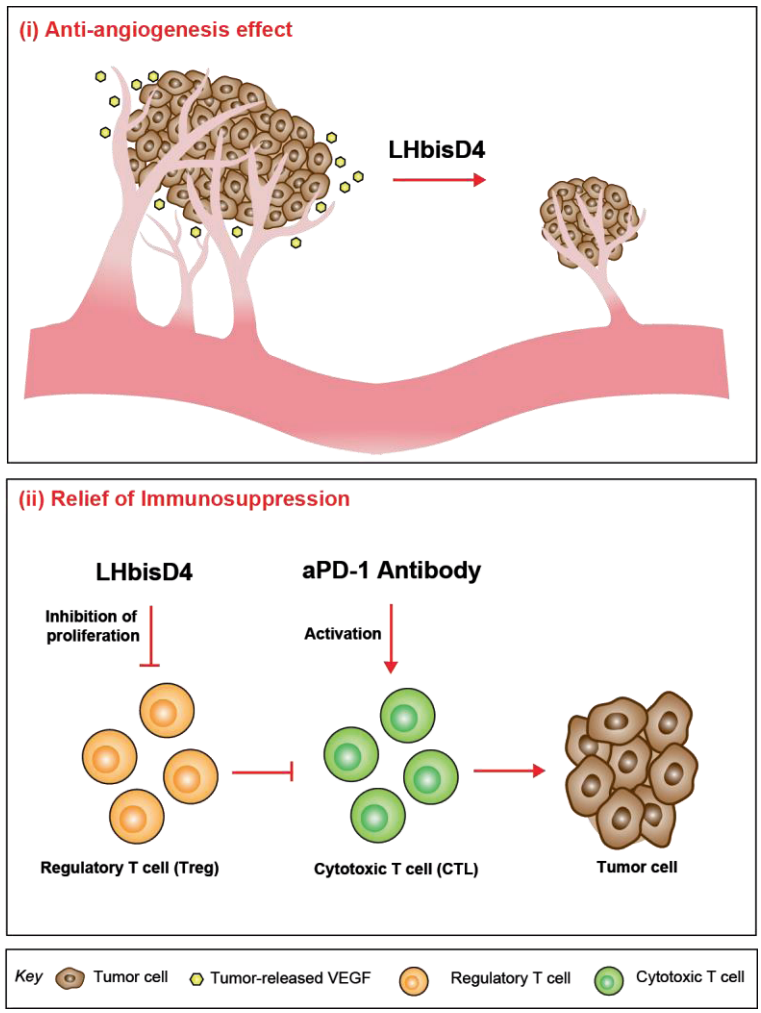


Figure 12. Schematic diagram of combination

5. Conclusions

Previously, we developed LHbisD4 as an orally active anti-angiogenic drug. We hypothesized in this study that LHbisD4 can exert not only an anti-angiogenic effect but also immunomodulatory effect by targeting the VEGF/VEGFR2 axis. Through various *in vitro* and *in vivo* experiments, we demonstrated that LHbisD4 can successfully suppress the process of tumor angiogenesis by interfering with the VEGF/VEGFR2 axis. Also, combination therapy of LHbisD4 and αPD-1 antibody diminished tumor growth in B16F10 bearing mice, which resulted in enhanced anti-tumor efficacy of anti PD-1 antibody. Through this study, we strongly suggest LHbisD4 as a powerful anti-angiogenic drug as well as an effective immunomodulatory agent, which can potentiate the efficacy of immune checkpoint inhibitors.

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Abstract (Kor)

종양 신생혈관생성은 종양 성장에 주요한 원인 으로, 지난 십 년간 주목을 받아왔다. 본 연구에서는 종양의 신생혈관생성뿐 아니라 조절 T 세포 (Regulatory T cell)의 증식을 억제하여 면역반응을 조절할 수 있다는 특징에 착안하여 VEGF-A (Vascular endothelial growth factor)을 표적화하는 약물을 사용했다. 저분자량헤파린 (LMWH)과 데옥시콜산 (Deoxycholic acid)을 결합시킨 LHbisD4 는 경구로 복용이 가능한 합성물질로, 종양신생혈관생성의 신호전달에 중요한 작용을 하는 혈관내피세포생성인자를 특이적으로 억제하여 종양혈관의 성장을 억제한다. 또한, 혈관내피세포성장인자가 면역반응을 조절하는 중요한 요소인 조절 T 세포 (Regulatory T cell)의 증식을 억제하여 면역반응을 조절할 수 있다는 특징에 착안하여, LHbisD4 가 종양신생혈관생성을 억제함과 동시에 항암면역반응을 촉진시킬 수 있다는 가정을 세웠다.

본 연구에서 Surface plasmon resonance (SPR)을 통해 LHbisD4 가 LMWH 에 비해 VEGF-A 에 대해 높은 친화도를 갖는 것과, 웨스턴블롯을 통해 LHbisD4 가 VEGF-A 에 의한 VEGFR-2 인산화를 억제하는 것을 규명하여 VEGF-A 표적 약물로서의 기능을 입증했다. LHbisD4 를 처리한 Human umbilical vein endothelial cell (HUVEC)의 증식, tube formation, spheroid sprouting 이 억제되는 것을 확인했다. 마지막으로 동물 모델에서 종양 성장억제율이 복합투여군이 대조군에 비해 78.2% 로 강력한 항암효과를 보였다.

위의 실험결과들이 LHbis4 와 항 PD-1 항체를 복합투여하여 종양 신생혈관생성을 억제하는 동시에 항암 면역반응을 상승시켜 항암작용을 향상시킬 수 있음을 뒷받침한다.

주요어: 종양신생혈관생성, 혈관내피세포생성인자, 조절 T 세포, 면역치료, PD-1

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