Stepwise inhibition of T cell recruitment at post-capillary venules by orally active desulfated heparins in inflammatory arthritis

Hasan Al Faruque¹,², Jin Hee Kang¹,², Seung Rim Hwang³, Shijin Sung¹,², Md. Mahmudul Alam¹,², Keum Hee Sa¹,², Eon Jeong Nam¹, Young Ro Byun⁴, Young Mo Kang¹,²*

¹ Division of Rheumatology, Department of Internal Medicine, Kyungpook National University School of Medicine, Daegu, South Korea, ² Department of Biochemistry and Cellular Biology, Kyungpook National University School of Medicine, Daegu, South Korea, ³ College of Pharmacy, Chosun University, Gwangju, South Korea, ⁴ Research Institute of Pharmaceutical Sciences, College of Pharmacy, Seoul National University, Seoul, South Korea

* ymkang@knu.ac.kr

Abstract

Identification of the structure-function relationship of heparin, particularly between 2-O-, 6-O-, and N-sulfation and its anticoagulant or anti-inflammatory activities, is critical in order to evaluate the biological effects of heparin, especially in conjunction with modifications for oral formulation. In this study, we demonstrated that removal of 2-O, 6-O, or N-desulfation and their hydrophobic modifications have differential effects on the blocking of interactions between sLeX and P-and L-selectins, with highest inhibition by 6-O desulfation, which was consistent with their in vivo therapeutic efficacies on CIA mice. The 6-O desulfation of lower molecular weight heparin (LMWH) retained the ability of LMWH to interfere with T cell adhesion via selectin-sLeX interactions. Furthermore, 6DSHbD coated on the apical surface of inflamed endothelium directly blocked the adhesive interactions of circulating T cells, which was confirmed in vivo by suppressing T cell adhesion at post-capillary venular endothelium. Thus, in series with our previous study demonstrating inhibition of transendothelial migration, oral delivery of low anticoagulant LMWH to venular endothelium of inflamed joint tissues ameliorated arthritis by the stepwise inhibition of T cell recruitment and provides a rationale for the development of modified oral heparins as innovative agents for the treatment of chronic inflammatory arthritis.

Introduction

T cells play a critical role in the pathogenesis of rheumatoid arthritis (RA) evidenced by the genetic association with major histocompatibility complex class II alleles and the T cell infiltrates within arthritic tissues [1, 2]. Recruitment of effector/memory T cells to specific sites of inflammation is an essential part of the immune response in chronic inflammatory disorders. As T cells contact activated endothelial cells at inflamed tissues, process of rolling, firm
adhesion, and transendothelial migration occur through interaction between adhesion molecules, including selectins, intercellular adhesion molecules (ICAMs) and vascular cell adhesion molecules (VCAMs). Guidance cues, such as adhesion molecules and chemokines play a critical role in regulating T cell extravasation and infiltration in tissues during inflammatory response [3]. Thus, therapeutics that alter cell migration represent a particularly promising class of the new anti-inflammatory drug, such as anti-α4 integrin monoclonal antibody [4] and mimetics of sialyl-LewisX (sLeX) [5, 6].

Heparin is a highly sulfated, linear polysaccharide composed of alternating units of hexuronic acid and glucosamine [7]. In addition to its well-established anticoagulant activity, mediated by high-affinity binding to antithrombin via a unique pentasaccharide sequence, heparin has been proposed to play a regulatory role in limiting inflammation [8]. Indeed, therapeutic efficacy in clinical trials of patients with inflammatory disorders [9, 10] may support the potential anti-inflammatory effects of heparin.

Several mechanisms have been proposed to explain the anti-inflammatory activity of heparin [11]. The therapeutic effects of heparin have been mainly attributed to its ability to inhibit the interaction between leukocytes and activated endothelial cells (ECs) and to neutralize inflammatory mediators, such as chemokines and growth factors at the site of inflammation [12–14]. We have shown that 6-O desulfation of lower molecular weight heparin (LMWH) conjugated with deoxycholic acid inhibits transmigration of T cells through activated ECs and inhibits recruitment of T cells into inflamed arthritis tissues [15]. However, the efficacy of desulfation of other sites of LMWH on the anti-inflammatory functions has not been reported yet. Furthermore, regulatory mechanism of modified heparins on the direct interaction between ECs at post-capillary venules and T cells has not been fully elucidated.

The size and charge of heparin, however, are generally accepted to preclude absorption from the gastrointestinal tract, and make parenteral administration a necessity (NRI 2002). A variety of formulation and enhancing strategies to increase oral bioavailability of heparin have been investigated [16–19]. A lipidation strategy which involves conjugation of deoxycholic acid (DOCA) to facilitate its transport through the intestinal epithelium has been successfully applied to desulfated LMWH in our previous reports [15, 20]. Thus, identification of modified LMWHs that exert higher anti-inflammatory efficacy as well as lower anti-coagulant activity offers not only an insight into the mechanisms of heparin action, but also significant potential for development of oral agents. In this study, we investigated whether desulfated LMWHs at different sites of the disaccharide unit accompanied by conjugation with DOCA modulate the T-cell adhesion on ECs during stepwise recruitment of T cells and whether they are differentially effective in suppressing disease activity in inflammatory arthritis.

### Materials and methods

#### Reagents and antibodies

The sources of reagents are as follows: protease-free bovine serum albumin (BSA, Miles Inc., Kankakee, IL), polyacrylamide-sialyl LewisX (PAA-sLeX; Glycotech, Rockville, MD), carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR), bovine type II collagen (CII), complete and incomplete Freund’s adjuvants (CFA and IFA, Chondrex, Redmond, WA), Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA), phytohemagglutinin (PHA; GibcoBRL, Rockville, MD), recombinant human IL-2 (Chiron, Amsterdam, NL), or mouse IL-2 (eBioscience, San Diego, CA). Recombinant human P-selectin-Fc chimera, TNF-α, and SDF-1α were purchased from R&D Systems (Minneapolis, MN). EDTA, Triton X-100, p-nitrophenyl N-acetyl-β-D-glucosaminide, Mayer’s H&E, protein A, and type IV

---

**Abbreviations:** 2DSHbD, 2-O desulfated-LMWH-bisdeoxycholic acid; 6DSHbD, 6-O desulfated-LMWH-bisdeoxycholic acid; CAI, clinical arthritis index; CFSE, Carboxyfluorescein succinimidyl ester; CIA, collagen-induced arthritis; DOCA, deoxycholic acid; DSFC, dorsal skinfold chamber; HbD, LMWH-bisdeoxycholic acid; HUVEC, human umbilical vein endothelial cell; ICAM, intercellular adhesion molecule; IL-2, interleukin-2; LMWH, lower molecular weight heparin; NDSHbD, N-desulfated N-acetylated-LMWH-bisdeoxycholic acid; RA, rheumatoid arthritis; SDF-1α, stromal cell-derived factor-1α; sLeX, sialyl-LewisX; TNF-α, tumor necrosis factor-α; VCAM, vascular cell adhesion molecule.

**Competing interests:** The authors have declared that no competing interests exist.

---

**PLOS ONE | https://doi.org/10.1371/journal.pone.0176110**

April 18, 2017 2 / 16
collagenase were purchased from Sigma-Aldrich (Saint Luis, MO). Antibodies against human CD3ε (clone 14-2C11; eBioscience) and P-selectin (clone 9E1) were used.

**Heparin synthesis**

LMWH-bisDOCA (HbD), 2-O desulfated-HbD (2DSHbD), 6DSHbD and N-desulfated N-acetylated-HbD (NDSHbD) were synthesized as previously described [15, 20]. Briefly, N-deoxycholyethylamine (DOCA-NH2) or N-bisdeoxycholyethylamine (bisDOCA-NH2) was chemically conjugated to the carboxylic groups of LMWH, 2DS-LMWH, 6DS-LMWH and NDSHbD. To activate carboxylic groups, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride was added to dissolved LMWH, 2DS-LMWH, 6DS-LMWH, or NDSH-LMWH in formamide, DOCA-NH2 or bisDOCA-NH2 in formamide and/or dimethylformamide was added dropwise. The mixture was washed in cold ethanol after a 12 hours reaction. A Coatest anti-factor Xa (FXa) chromogenic assay (Chromogenix, Milano, Italy) was used to determine the anticoagulant activity. Sulfuric acid assay was used to determine the conjugation ratios of DOCA-NH2 or bisDOCA-NH2 to LMWH, 2DS-LMWH, 6DS-LMWH, or NDS-LMWH, respectively [21]. Structures of modified heparins were shown in Fig 1.

**ELISA inhibition assay**

ELISA inhibition assays were performed as previously described [13]. Briefly, PAA-sLeX was coated (200 ng/well) onto ELISA plates (Costar, Cambridge, MA) by overnight incubation and then blocked with 1% protease-free BSA for 1 hour at 4˚C. Heparin, buffer (positive control), or 10 mM NaEDTA (negative control) were preincubated with selectin-IgG-Fc chimera. Peroxidase-conjugated goat anti-human IgG (Jackson ImmunoResearch Laboratories) and the mixtures were added to coated wells. After incubation at 4˚C for 4 hours, the plate was developed with O-phenylenediamine dihydrochloride. The absorbance was measured at 405 nm with a microplate reader (Molecular Device). The data were converted into percentages by using the formula: 
\[
\frac{[(\text{mean of duplicates})-(\text{mean of negative control})]/[(\text{mean of positive control})-(\text{mean of negative control})]}{100}.
\]

**Cell culture**

Human T cells were prepared by negative selection using magnetic bead separation (Miltenyi Biotec, Auburn, CA) following Ficoll-density gradient separation of peripheral blood mononuclear cells. This study was approved by the institutional review board of Kyungpook National University Hospital and written informed consent was obtained. Human umbilical vein endothelial cell (HUVECs, CRL-1730 ATCC) were cultured in EGM-2 medium and stimulated with TNF-α (10 ng/mL) for 16 hours before static and dynamic studies. Murine T cells were prepared by negative selection with magnetic separation kit (Miltenyi Biotec) from spleenocytes followed by stimulation with PHA and murine IL-2 (20 ng/mL).

**T cell adhesion assay under static conditions**

The T cell adhesion assay was performed as previously described with some minor modifications [22]. Briefly, recombinant human P-selectin-IgG-Fc chimera (0.1 µg/mL) or PAA-sLeX (0.1 µg/mL) was coated onto ELISA plates overnight at 4˚C. After blocking with 1% endotoxin-free BSA, T cells (2 × 10^5/well) were incubated in coated wells containing heparin derivatives or EDTA (5 mM) for 2 hours. Adherent T cells were lysed using 1% Triton X-100 and p-nitrophenyl-N-acetyl-β-D-glucosaminide (3.75 mM) in citrate buffer. The absorbance was measured at 405 nm using a microplate reader.
Analysis of lymphocyte motion under shear flow

Laminar flow adhesion assays were performed as described previously [23]. Immobilized P-selectin (1 µg/ml) bound to protein A (8 µg/ml) and TNF-α-stimulated confluent HUVECs were incubated on a 35-mm polystyrene dish. Effector T cells (1 × 10^6/ml) preincubated with 6DSHbD or EDTA (5 mM) were perfused at 1.8 dynes/cm² and recorded in high-power fields by videomicroscopy. Data were processed using MTrackJ (an ImageJ plug-in software created by Erik Meijering, Erasmus MC, Rotterdam, NL). Adherent cells were defined as cells that did not move during a 10 seconds interval, and interacting cells were defined as cells that interacted with the P-selectin or HUVEC monolayer for at least 1 second. Rolling flux and velocity were determined by counting the number of rolling cells and dividing the distance by the time within a field of view.

Animal experiments

All animal care and experimental procedures were approved by the Kyungpook National University Institutional Animal Care and Use Committee (Approval Number: KNU 2012–35) and conducted in accordance with the institutional protocol for animal welfare. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments.
Dorsal skin fold chambers (DSFC) were implanted on the back skin of CIA mice by surgical
in vivo
The hind paws of randomly chosen CIA mice from each treatment group were homogenized
Semi-quantitative RT-PCR
in lysis reagent (Easy-spin; Intron Biotech, Sungnam, Korea). Total RNA was extracted using
an RNeasy extraction kit (Intron Biotech) from the joint tissues after removing the skin of the
hind paws. We employed 1 μg of total RNA for reverse transcription using oligo-dT and the
PrimeScript 1st strand cDNA synthesis kit (Takara Bio, Shiga, Japan). Relative transcript levels
for inflammatory mediators were quantified by real-time semiquantitative PCR, which was
performed using primers, Taqman probes, and a LightCycler 480 system (Roche Diagnostics,
Mannheim, Germany). The expression of inflammatory mediators was normalized to that of a
target reference gene (18s ribosome RNA). The Taqman probes and oligonucleotides used are
listed as follows: IL-1β: forward (F) (5′-TGTAATGAAAGACGGCACACC-3′), reverse (R) (5′-
TCTTCTTTGGATATTGGTGG-3′), and probe (5′-CTGCTTCC-3′); IL-6: F (5′-GAGAAAAAG
ATGTGCAATGGC-3′), R (5′-CCAGTTTGGTAGCATCCACA-3′), and probe (5′-TTCCCTC
TG-3′); TNF-α: F (5′-CTGTAGCCACCTGCTAAGC-3′), R (5′-TTGAGATCCACTGCGCTG-
TG-3′), and probe (5′-ACGTCTGAG-3′); MMP-1: F (5′-TTGTTTCACAAACGAGACC-3′), R
(5′-GCCCAAGTGTAGTATGTTCTCCA-3′), and probe (5′-CATCCAGG-3′); MMP-3: F (5′-
TGTTTTTGATGCGATCACC-3′), R (5′-GATTTGGGCAAAAGTGCC-3′), and probe (5′-
GGGAGAAC-3′); VCAM-1: F (5′-TGGTGAATGGAATCTGACCC-3′), R (5′-CCCGATGTTG
GTTTCCCTT-3′), and probe (5′-AGGCGAGG-3′); RANKL: F (5′-TGAAGCACACACTACCTG
ACTCCTG-3′), R (5′-CCACATGTGTTTCAGTCC-3′), and probe (5′-GGGAGATG-3′); 18s ribosome RNA: F (5′-AAATCAGTTATGTTGCTTCC-3′), R (5′-GCTCTAAGATTACCAAGTTC-3′), and probe (5′-TCTCTCCTCC-3′).

Anti-arthritic effects of oral desulfated heparins

in vivo
anticoagulant activity was measured using prothrombin time (PT) and activated partial
thromboplastin time (aPTT) after oral treatment with heparin derivatives (Chemon, Suwon,
Korea) in CIA mice (control, n = 4; NDSHbD, n = 3; 2DSHbD, n = 3; 6DSHbD, n = 3).

Semi-quantitative RT-PCR

The hind paws of randomly chosen CIA mice from each treatment group were homogenized
in lysis reagent (Easy-spin; Intron Biotech, Sungnam, Korea). Total RNA was extracted using
an RNeasy extraction kit (Intron Biotech) from the joint tissues after removing the skin of the
hind paws. We employed 1 μg of total RNA for reverse transcription using oligo-dT and the
PrimeScript 1st strand cDNA synthesis kit (Takara Bio, Shiga, Japan). Relative transcript levels
for inflammatory mediators were quantified by real-time semiquantitative PCR, which was
performed using primers, Taqman probes, and a LightCycler 480 system (Roche Diagnostics,
Mannheim, Germany). The expression of inflammatory mediators was normalized to that of a
target reference gene (18s ribosome RNA). The Taqman probes and oligonucleotides used are
listed as follows: IL-1β: forward (F) (5′-TGTAATGAAAGACGGCACACC-3′), reverse (R) (5′-
TCTTCTTTGGATATTGGTGG-3′), and probe (5′-CTGCTTCC-3′); IL-6: F (5′-GAGAAAAAG
ATGTGCAATGGC-3′), R (5′-CCAGTTTGGTAGCATCCACA-3′), and probe (5′-TTCCCTC
TG-3′); TNF-α: F (5′-CTGTAGCCACCTGCTAAGC-3′), R (5′-TTGAGATCCACTGCGCTG-
TG-3′), and probe (5′-ACGTCTGAG-3′); MMP-1: F (5′-TTGTTTCACAAACGAGACC-3′), R
(5′-GCCCAAGTGTAGTATGTTCTCCA-3′), and probe (5′-CATCCAGG-3′); MMP-3: F (5′-
TGTTTTTGATGCGATCACC-3′), R (5′-GATTTGGGCAAAAGTGCC-3′), and probe (5′-
GGGAGAAC-3′); VCAM-1: F (5′-TGGTGAATGGAATCTGACCC-3′), R (5′-CCCGATGTTG
GTTTCCCTT-3′), and probe (5′-AGGCGAGG-3′); RANKL: F (5′-TGAAGCACACACTACCTG
ACTCCTG-3′), R (5′-CCACATGTGTTTCAGTCC-3′), and probe (5′-GGGAGATG-3′); 18s ribosome RNA: F (5′-AAATCAGTTATGTTGCTTCC-3′), R (5′-GCTCTAAGATTACCAAGTTC-3′), and probe (5′-TCTCTCCTCC-3′).

in vivo
intra-vital microscopic analysis

Dorsal skin fold chambers (DSFC) were implanted on the back skin of CIA mice by surgical
intervention according to previously described method with a minor modification [27, 28].
After chamber implantation, mice were kept in separate cages providing adequate amount of
food and water. A recovery period of 7 days between implantation of DSFC and quantitative
image analysis was allowed. CFSE labeled effector T cells (1 x 10⁷ cells) were suspended in PBS
and 200 μl of suspension was injected in the tail vein of mice. Number of adherent T cells at
the post-capillary venules was recorded with epifluorescence microscopy (OV-100, Olympus Co, Tokyo, Japan).

Statistics
Statistical analysis was performed using SPSS version 12 (SPSS Inc., Chicago, IL). The difference between groups was analyzed by Student’s t-test. For the comparison of the difference between treatment groups during multiple time points, repeated measures ANOVA with Tukey’s post hoc test was performed. P value less than 0.05 was considered significant. Data are presented as the mean ± SEM unless otherwise indicated.

Results
Desulfations of LMWH conjugated with deoxycholic acid on the inhibition of selectin-sLe\(^{X}\) interaction
To examine the antiadhesive role of modified LMWHs, we prepared regioselectively desulfated LMWHs and their conjugates with bisDOCA. The anti-FXa activity of 6DSHbD and NDSHbD was nearly completely abolished compared to that of LMWH, while 2DSHbD retained anti-FXa activity partially (7.1% relative to that of fraxiparine). We then tested their inhibitory efficacy on P-selectin-sialyl Lewis\(^{X}\) (sLe\(^{X}\)) interaction (Fig 2A). Binding of P-selectin-IgG chimera to immobilized polyacrylamide-sLe\(^{X}\) was most efficiently inhibited by 6-O desulfated heparin compared with N-desulfated, 2-O desulfated, and non-desulfated LMWHs. IC\(_{50}\) value for inhibition of P-selectin-sLe\(^{X}\) interaction by 6DSHbD was 20.68 ± 6.75 μg/ml, while those of 2DSHbD, NDSHbD, and LMWH were 507.31 ± 29.50 μg/ml, 68.71 ± 5.20 μg/ml, and 101.67 ± 44.50 μg/ml. L-selectin-sLe\(^{X}\) binding, however, was inhibited to a similar extent by all

![Fig 2. Inhibition of selectin-sLe\(^{X}\) interaction by desulfated heparins.](https://doi.org/10.1371/journal.pone.0176110.g002)
these modified heparins (Fig 2B, IC_{50}: 3.06 ± 0.29, 8.20 ± 0.70, 8.56 ± 0.85, and 8.76 ± 0.30 μg/ml for LMWH, 2DSHbD, 6DSHbD, and NDSHbD, respectively). These data showed that removal of 6-O, 2-O, and N-sulfations and their hydrophobic modifications with deoxycholic acid had differential effects on the inhibition of interactions between sLe^X and P- and L-selectins, thereby necessitating the comparison of in vivo anti-inflammatory effects of these materials in inflammatory arthritis models.

**Therapeutic efficacy of oral desulfated heparins in collagen-induced arthritis**

We next determined whether bisDOCA conjugated LMWHs with distinct desulfations have differential efficacy on the inflammation using murine CIA model which has T cell-mediated pathophysiology [29, 30]. In this murine model, joint symptoms became apparent between days 21 and 23 and peak between days 40 and 45 after the first immunization. We treated mice with CII to induced CIA and then administered modified heparins via gavage beginning from day 23 after the first immunization for the following days until day 45 (Fig 3). Anticoagulant

---

**Fig 3. Therapeutic efficacy of oral desulfated heparins in murine collagen-induced arthritis.** (A-B), CIA mice were treated with daily oral administration of 2DSHbD, 6DSHbD, and NDSHbD (1 mg/kg/day) or treated with a negative control (PBS, oral daily) and a positive control (Methotrexate 1mg/kg, two times/week, intraperitoneal). Efficacy of treatment was analyzed by CAI (A) and incidence of arthritic paws (B). Data represent the mean ± SEM (n = 8 for each group). *p < 0.05. (C), Transcript level of inflammatory mediators in the joint tissues from control and desulfated heparin-treated CIA mice. Semiquantitative real-time PCR was performed using Taqman probes and analyzed using a LightCycler480 system. Data represent the mean ± SEM. *p < 0.05 versus control.

https://doi.org/10.1371/journal.pone.0176110.g003
activities measured by PT and aPTT revealed no significant difference between the control group and modified heparin treated groups (Table 1).

Upon measurement of CAI and incidence of arthritis on the paws, mice treated with all three modified heparins (1mg/kg/day, p.o.) showed significantly reduced severity of arthritis compared with methotrexate (1 mg/kg, two times a week, i.p.) and control (PBS, daily, p.o.). Among these modified heparins, 6DSHbD ameliorated the arthritis more effectively compared to 2DSHbD and NDSHbD (Fig 3A and 3B, p < 0.05).

To characterize effects of modified heparins on the inflammatory response within joint tissues, we quantified transcripts of inflammatory mediators from the whole extracts of arthritic tissues. Suppression of inflammation, as evidenced by reduced inflammatory transcripts within joints, was found in CIA mice treated with all three modified heparins compared to controls (Fig 3C). These data showed that 6DSHbD was most effective in ameliorating severity of CIA after oral administration as well as it retained the least anticoagulant activity among different desulfated LMWHs. Thus, we chose 6DSHbD for subsequent experiments in this study.

### Inhibition of selectin-mediated T cell adhesion by 6DSHbD in static and dynamic conditions

P-selectin glycoprotein ligand-1 (PSGL-1) plays a central role in the trafficking of lymphocytes to areas of inflammation by direct interaction with P- and L-selectins via sLeX and a sulfate group [31, 32]. To determine whether 6DSHbD modulates the selectin-mediated adhesive processes, we tested the adhesion of T cells to either P-selectin-IgG chimera or PAA-sLeX immobilized on plastic plates. In contrast to the results on the selectin-sLeX interaction, the adhesion of T cells to P-selectin was inhibited by 6DSHbD to a similar extent compared with LMWH (Fig 4A). Furthermore, the inhibition of sLeX-mediated T cell adhesion, which indicates sLeX-L-selectin interaction, was greater by 6DSHbD than by LMWH (IC50: 6.26 ± 3.05 μg/mL vs. 47.93 ± 20.79 μg/mL for 6DSHbD vs. LMWH, respectively, p < 0.05) (Fig 4B).

To more closely approach the physiological situation, we used phase-contrast videomicroscopy to study the effect of modified heparins on the adherence of T cells to immobilized P-selectin under physiological laminar shear flow. Approximately 47.8 ± 13.5 cells/mm² contacting P-selectin bound to protein A adhered to the plate surface. In comparison, 29.1 ± 2.8 cells/mm² treated with 6DSHbD adhered to P selectin-coated surface (Fig 4C, p < 0.05). The number of interacting T cells also was significantly reduced by treatment with 6DSHbD compared to control (Fig 4D). However, rolling flux and velocity were not influenced by 6DSHbD when compared to control (Fig 4E and 4F). These findings showed that 6-O-desulfation of LMWH

### Table 1. Anticoagulant activities after oral treatment with heparin derivatives in collagen-induced arthritis mice.

| Heparin derivatives | Anticoagulation activities |
|---------------------|---------------------------|
|                     | PT (sec) | aPTT (sec) |
| Control (n = 4)     | 7.18 ± 0.41 | 20.30 ± 1.36 |
| NDSHbD (n = 3)      | 7.47 ± 0.12 | 20.23 ± 0.76 |
| 2DSHbD (n = 3)      | 7.53 ± 0.15 | 19.97 ± 0.49 |
| 6DSHbD (n = 3)      | 7.30 ± 0.20 | 19.03 ± 1.29 |

NDSHbD, N desulfated LMWH conjugated with bis-DOCA; 2DSHbD, 2-O desulfated LMWH conjugated with bis-DOCA; 6DSHbD, 6-O desulfated LMWH conjugated with bis-DOCA. LMWH, Low molecular weight heparin; DOCA, deoxycholic acid; PT, Prothrombin time; aPTT, Activated partial thromboplastin time.

https://doi.org/10.1371/journal.pone.0176110.t001
retained the ability of LMWH to interfere with the adhesion between T cells and endothelial cells via selectin-sLeX interactions in both static and dynamic conditions.

**Inhibition of T cell adhesion on activated endothelial cells by 6DSHbD in static and dynamic conditions**

The early steps that promote recruitment of effector/memory T cells to non-lymphoid tissues and sites of inflammation include selectin-mediated rolling, chemokine-triggered activation, and integrin-dependent arrest on the endothelial surface [32]. We have shown that 6DSHbD displayed greater binding to TNF-α-stimulated HUVECs compared to unstimulated cells, followed by internalization into ECs [15]. We examined whether surface-bound modified...
heparin can directly block the adhesion of T cells to HUVEC monolayers under both static and dynamic conditions.

Under static conditions, 6DSHbD-Cy5.5 (red) bound on the surface of TNF-α-stimulated HUVECs in a dose-dependent manner. The adhesion of T cells labeled with CFSE (green) on the HUVECs was prevented from the surface of endothelial cells where 6DSHbD-Cy5.5 was accumulated (Fig 5A). The number of T cells adherent to the surface of HUVECs decreased significantly following treatment with a higher concentration (30 μg/ml) of 6DSHbD (Fig 5B).

The efficiency of T cell recruitment, as determined by the conversion from rolling to arrest in post-capillary venules, was recapitulated on TNF-α-stimulated HUVECs in the parallel-plate flow chamber [23]. Rolling and adhesive interactions of T cells with HUVEC monolayers were examined under defined laminar flow. The average rolling velocity of T cells on activated HUVECs under a flow of 1.8 dynes/cm² was significantly lower compared with that on unstimulated HUVECs. After treatment with 6DSHbD, both the average rolling velocity and cumulative frequency over the rolling velocity of T cells were slightly elevated compared to those of untreated cells, although the differences were not statistically significant (Fig 5C and 5D). The number of stably arrested T cells on stimulated endothelial cells was significantly decreased by 6DSHbD treatment (426 ± 82 vs. 291 ± 44 cells/mm², p < 0.05) (Fig 5E). These findings show that 6DSHbD coated on the apical surface of inflamed endothelium directly interferes with the adhesive interactions of circulating T cells with endothelial cells.

**Reduced T cell adhesion on the activated venular endothelium under the intra-vital microscopy**

To evaluate the efficacy of oral 6DSHbD on the diapedesis of T cells through the post-capillary venules *in vivo*, we prepared a modified DSFC using small round chamber covered with a removable glass and implanted this chamber on the back skin of DBA1/J mice. Mice were treated orally with 6DSHbD (10 mg/kg) for 3 days, followed by intravenous injection of CFSE-labeled effector T cells (2 x 10⁶ cells). Prior to T cell transfer, cover glass was removed and the exposed vessels were stimulated with TNF-α (10 ng/ml) and a chemokine (SDF-1α, 10 μg/ml) for 4 hours (Fig 6A). Venules with diameter between 40 and 80 μm were selected and the adherent and transmigrating T cells (green) were recorded using fluorescent videomicroscopy (Fig 6B). The number of adherent T cells on the venular surface was significantly reduced in 6DSHbD treated mice (Fig 6C). These findings confirmed that 6DSHbD, accumulated on the activated endothelium, directly inhibited the adhesion of effector T cells on the endothelial cell surface at the post-capillary venules *in vivo*.

**Discussion**

Identification of the structure-function relationship of heparin, particularly between 2-O-, 6-O-, and N-sulfation and its anticoagulant or anti-inflammatory activities, is critical in order to evaluate the biological effects of heparin, especially in conjunction with modifications for oral formulation [8, 13]. In the present study, we demonstrated that removal of 2-O, 6-O, and N-desulfations and their hydrophobic modifications have differential effects on the blocking of interactions between sLeX and P-and L-selectins, with highest inhibition by 6-O desulfation, which was consistent with *in vivo* therapeutic efficacy on CIA mice. The 6-O desulfation of LMWH retained the ability of LMWH to interfere with T cell adhesion via selectin-sLeX interactions. Furthermore, 6DSHbD coated on the apical surface of inflamed endothelium directly blocked the adhesive interactions of circulating T cells with activated endothelial cells, which was confirmed *in vivo* by suppressing T cell adhesion at post-capillary venules.
Fig 5. Inhibition of T cell adhesion on activated ECs by 6DSHbD in static and dynamic conditions. (A–B), Unstimulated or TNF-α-stimulated HUVEC monolayers were incubated with 6DSHbD-Cy5.5 (μg/ml, red) for 30 minutes and co-cultured with CFSE-labeled T cells (green) in a static condition. Fluorescence microscopy of the adherent T cells with HUVECs (A) and the number of adherent T cells was counted in 5 random high-power fields (B). Scale bars, 100 μm. (C–E), Unstimulated or TNF-α-stimulated HUVEC monolayers were treated with 6DSHbD and placed in a parallel plate-flow chamber device. T cells were allowed to interact with HUVEC monolayers at a shear stress of 1.8 dynes/cm². The rolling velocity of T cells (C), cumulative frequency of the rolling velocities of T cells (D), and the number of adherent T cells on HUVECs (E) were analyzed.

https://doi.org/10.1371/journal.pone.0176110.g005
The 2-O- and 3-O-sulfation provide favorable contacts between heparin and antithrombin, and 6-O-sulfate group appears to grant increased catalytic efficacy to anti-thrombin [33]. We observed that 6-O-desulfation and N-desulfation of LMWH abrogated anticoagulant activity, whereas 2-O-desulfation produced only a partial reduction in anticoagulant activity, which is consistent with studies on the anticoagulant efficacy of regioselectively desulfated natural heparins [13, 34]. Removal of the anticoagulant function of LMWH, however, did not affect its ability to block T cell-endothelial cell adhesion via selectin-sLe\(^X\) interactions. Whereas the 6-O-sulfate of glucosamine is important for binding to selectin [13] and FGF2 [35], 2-O- and/or 3-O-desulfation of unfractionated heparin inhibits the adhesion of monocytes to selectins [13] or the receptor for advanced glycation endproducts (RAGE) [36], as well as the adhesion of neutrophils to endothelial cells [34]. Partial 6-O-desulfation may retain enough anionic charge to mediate electrostatic interactions with sufficient affinity to preserve the biologic properties of heparin [37].

In murine CIA model, all three desulfated heparins were more effective for the treatment of arthritis compared to methotrexate, which is a standard disease modifying anti-rheumatic drug. Among desulfated heparins, 6DSHbD showed highest therapeutic efficacy. These data suggest an effect of 6-O desulfation on anti-inflammatory effects which could be direct and/or indirect. Data in Fig 2 argue for a direct effect by more effective blocking of P-selectin-sLe\(^X\) binding. Previous studies revealed higher bioavailability of 6DSHbD compared to 2DSHbD. Furthermore, intracellular accumulation of hydrophobically modified desulfated heparins may result in the differential regulation on signaling pathways that require further investigation [15].

Fig 6. Reduced T cell adhesion to the post-capillary venular endothelium on intravital microscopy. (A) Image of implanted dorsal skinfold chamber on back of DBA/1J mouse. (B) Representative images of intravital microscopy of CFSE-labeled T cell adhesion on endothelium at post-capillary venules in vivo. (C) Quantification of adherent T cells on the endothelial surface in control (n = 3) and 6DSHbD treated mice (n = 3). Data are mean ± SEM from 3 independent experiments. *p < 0.05.
Unfractionated heparin binds to and is internalized by endothelial and vascular smooth muscle cells and is involved in the regulation of signaling pathways [38, 39]. Heparin is cleared by both rapid saturable and slower, first-order mechanisms. The rapid saturable clearance phase following intravenous injection may be due to the binding of heparin to endothelial cells and its subsequent uptake [40]. In contrast, LMWH displays reduced binding to HUVECs, which may result in its clearance by primarily nonsaturable or renal mechanisms [40]. We have previously shown that 6DSHbD internalized into endothelial cells after binding to P-Selectin and VCAM-1 which leads to the inhibition of transcellular migration of T cells by blocking RhoA GTPase activation [15]. In the present study, we further demonstrated that 6DSHbD bound on the surface of activated ECs directly hampered the adhesion of T cells on the ECs, thus reducing the chance to be recruited into inflamed joint tissues prior to internalization of 6DSHbD.

An essential step in this process is the adhesion of effector T cells to the endothelium of post-capillary venules—a complex multistep cascade of events mediated by adhesion receptors [41], such as P-selectin and VCAM-1, which varies depending on the target tissue and the inflammatory context. Inhibition of T cell homing to synovial tissue (ST) represents a potential strategy for the treatment of chronic inflammatory arthritis. Another critical step, we have shown, was that the oral administration of 6DSHbD resulted in its preferential accumulation within inflamed ST, particularly around post-capillary venules [15]. Moreover, reduced homing of effector T cells to inflamed ST that occurred in response to the oral 6DSHbD was dependent on P-selectin-mediated cellular accumulation of 6DSHbD [15]. However, there has been mechanistic gap between the accumulation of 6DSHbD and reduced homing of effector T cells, which may be explained either by the direct inhibition of T cell adhesion on ECs or by sequestration of chemokines [42, 43]. Actually, neutralization of multiple CC chemokines, which may also achieved by heparin especially via inhibition of oligomerization [44], resulted in a reduction of disease activity in an arthritis model through the retention of effector T cells [43, 45]. The results of the present study may fill this gap by clearly demonstrating that 6DSHbD directly inhibited adhesion of T cells on the endothelium of post-capillary venules by blocking the tethering and adhesion of T cells on 6DSHbD-coated surface of endothelium.

To our knowledge, this is the first report to show that oral delivery of low anticoagulant LMWH to venular endothelium of inflamed joint tissues protects from arthritis. This effect is mediated by the stepwise inhibition of T cell recruitment and provides a rationale for the development of modified oral heparins as innovative agents for the prevention and/or treatment of chronic inflammatory arthritis.

Author Contributions
Conceptualization: YRB YMK.
Formal analysis: JHK.
Funding acquisition: YMK.
Investigation: HAF JHK SRH SS MMA KHS.
Methodology: HAF JHK MMA.
Project administration: YRB YMK.
Resources: SRH JHK EJN YMK.
Supervision: YMK.
Validation: SS KHS.

Visualization: HAF SS.

Writing – original draft: HAF YMK.

Writing – review & editing: EJN YMK.

References

1. Firestein GS. Evolving concepts of rheumatoid arthritis. Nature. 2003; 423(6937):356–61. Epub 2003/05/16. https://doi.org/10.1038/nature01661 PMID: 12748655

2. McInnes IB, Schett G. Cytokines in the pathogenesis of rheumatoid arthritis. Nat Rev Immunol. 2007; 7(6):429–42. Epub 2007/05/26. https://doi.org/10.1038/nri2094 PMID: 17525752

3. Luster AD, Alon R, von Andrian UH. Immune cell migration in inflammation: present and future therapeutic targets. Nat Immunol. 2005; 6(12):1182–90. Epub 2005/12/22. https://doi.org/10.1038/ni1275 PMID: 16369557

4. Mackay CR. Moving targets: cell migration inhibitors as new anti-inflammatory therapies. Nat Immunol. 2008; 9(9):888–98. Epub 2008/08/20. https://doi.org/10.1038/ni.f.210 PMID: 18711436

5. Friedrich M, Bock D, Philipp S, Ludwig N, Sabat R, Wolk K, et al. Pan-selectin antagonism improves psoriasis manifestation in mice and man. Arch Dermatol Res. 2006; 298(6):345–51. Epub 2005/12/20. https://doi.org/10.1007/s00403-005-0626-0 PMID: 16962415

6. Kaneider NC, Leger AJ, Kuliopolus A. Therapeutic targeting of molecules involved in leukocyte-endothelial cell interactions. FEBS J. 2006; 273(19):4416–24. Epub 2006/08/08. https://doi.org/10.1111/j.1742-4658.2006.05441.x PMID: 16995349

7. Capilla I, Linhardt RJ. Heparin-protein interactions. Angew Chem Int Ed Engl. 2002; 41(3):391–412. Epub 2002/12/20. PMID: 12491369

8. Lever R, Page CP. Novel drug development opportunities for heparin. Nat Rev Drug Discov. 2002; 1(2):140–8. Epub 2002/07/18. https://doi.org/10.1038/nrd724 PMID: 12120095

9. Ahmed T, Garrigo J, Danta I. Preventing bronchoconstriction in exercise-induced asthma with inhaled heparin. N Engl J Med. 1993; 329(2):90–5. Epub 1993/07/08. https://doi.org/10.1056/NEJM199307083290204 PMID: 8510708

10. Chande N, McDonald JW, Macdonald JK. Unfractionated or low-molecular weight heparin for induction of remission in ulcerative colitis. Cochrane Database Syst Rev. 2008;(2):CD006774. Epub 2008/04/22. https://doi.org/10.1002/14651858.CD006774.pub2 PMID: 18425969

11. Young E. The anti-inflammatory effects of heparin and related compounds. Thromb Res. 2008; 122(6):743–52. Epub 2008/07/31. https://doi.org/10.1016/j.thromres.2008.06.026 PMID: 17727922

12. Koenig A, Norgard-Sumnicht K, Linhardt R, Varki A. Differential interactions of heparin and heparan sulfate glycosaminoglycans with the selectins. Implications for the use of unfractionated and low molecular weight heparins as therapeutic agents. J Clin Invest. 1998; 101(4):877–89. Epub 1998/03/21. https://doi.org/10.1172/JCI1509 PMID: 9466983

13. Wang L, Brown JR, Varki A, Esko JD. Heparin’s anti-inflammatory effects require glucosamine 6-O-sulfation and are mediated by blockade of L- and P-selectins. J Clin Invest. 2002; 110(1):127–36. Epub 2002/07/03. https://doi.org/10.1172/JCI14996 PMID: 12093896

14. Tyrrell DJ, Horne AP, Holme KR, Preuss JM, Page CP. Heparin in inflammation: potential therapeutic applications beyond anticoagulation. Adv Pharmacol. 1999; 46:151–208. Epub 1999/05/20. PMID: 10332503

15. Kang JH, Hwang SR, Sung S, Jang JA, Alam MM, Sa KH, et al. Intracellular delivery of desulfated heparin with bile acid conjugation alleviates T cell-mediated inflammatory arthritis via inhibition of RhoA-dependent transcellular diapedesis. J Control Release. 2014; 183:9–17. Epub 2014/03/25. https://doi.org/10.1016/j.jconrel.2014.03.029 PMID: 24657949

16. Jiao Y, Ubrich N, Marchand-Arvier M, Vigneron C, Hoffman M, Lecompte T, et al. In vitro and in vivo evaluation of oral heparin-loaded polymeric nanoparticles in rabbits. Circulation. 2002; 105(2):230–5. Epub 2002/01/16. PMID: 11790706

17. Son SJ, Bai X, Nan A, Ghandehari H, Lee SB. Template synthesis of multifunctional nanotubes for controlled release. J Control Release. 2006; 114(2):143–52. Epub 2006/07/28. https://doi.org/10.1016/j.jconrel.2006.06.004 PMID: 16870299

18. Thanou M, Nihot MT, Jansen M, Verhoef JC, Junginger HE. Mono-N-carboxymethyl chitosan (MCC), a polyampholytic chitosan derivative, enhances the intestinal absorption of low molecular weight heparin
anti-arthritic effects of oral desulfated heparins across intestinal epithelia in vitro and in vivo. J Pharm Sci. 2001; 90(1):38–46. Epub 2000/11/07. PMID: 11064377

19. Berkowitz SD, Marder VJ, Kosutic G, Baughman RA. Oral heparin administration with a novel drug delivery agent (SNAC) in healthy volunteers and patients undergoing elective total hip arthroplasty. J Thromb Haemost. 2003; 1(9):1914–9. Epub 2003/08/28. PMID: 12941031

20. Hwang SR, Seo DH, Al-Hilal TA, Jeon OC, Kang JH, Kim SH, et al. Orally active desulfated low molecular weight heparin and deoxycholic acid conjugate, 6ODS-LHzD, suppresses neovascularization and bone destruction in arthritis. J Control Release. 2012; 163(3):374–84. Epub 2012/10/09. https://doi.org/10.1016/j.conrel.2012.09.013 PMID: 23041275

21. Park JW, Jeon OC, Kim SK, Al-Hilal TA, Moon HT, Kim CY, et al. Anticoagulant efficacy of solid oral formulations containing a new heparin derivative. Mol Pharm. 2010; 7(3):836–43. Epub 2010/03/31. https://doi.org/10.1021/mp900319k PMID: 20349950

22. Nam EJ, Kang JH, Sung S, Sa KH, Kim KH, Seo JS, et al. A matrix metalloproteinase 1-cleavable composite peptide derived from transforming growth factor beta-inducible gene h3 potently inhibits collagen-induced arthritis. Arthritis Rheum. 2013; 65(7):1753–63. Epub 2013/03/20. https://doi.org/10.1002/art.37932 PMID: 23508269

23. Jones DA, McIntire LV, Smith CW, Picker LJ. A two-step adhesion cascade for T cell/endothelial cell interactions under flow conditions. J Clin Invest. 1994; 94(6):2443–50. Epub 1994/12/01. https://doi.org/10.1172/JCI117612 PMID: 7527432

24. McGrath JC, Drummond GB, McLachlan EM, Kilkenny C, Wainwright CL. Guidelines for reporting experiments involving animals: the ARRIVE guidelines. Br J Pharmacol. 2010; 160(7):1573–6. Epub 2010/07/24. https://doi.org/10.1111/j.1476-5381.2010.00873.x PMID: 20649560

25. Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG. Animal research: reporting in vivo experiments: the ARRIVE guidelines. Br J Pharmacol. 2010; 160(7):1577–9. Epub 2010/07/24. https://doi.org/10.1111/j.1476-5381.2010.00872.x PMID: 20649561

26. Nam EJ, Sa KH, You DW, Cho JH, Seo JS, Han SW, et al. Up-regulated transforming growth factor beta-inducible gene h3 in rheumatoid arthritis mediates adhesion and migration of synoviocytes through alpha v beta3 integrin: Regulation by cytokines. Arthritis Rheum. 2006; 54(9):2734–44. Epub 2006/09/02. https://doi.org/10.1002/art.22076 PMID: 16947382

27. Lehr HA, Leunig M, Menger MD, Nolte D, Messmer K. Dorsal skinfold chamber technique for intravital microscopy in nude mice. Am J Pathol. 1993; 143(4):1055–62. Epub 1993/10/01. PMID: 7692730

28. Palmer GM, Fontanella AN, Shan S, Hanna G, Zhang G, Fraser CL, et al. In vivo optical molecular imaging and analysis in mice using dorsal window chamber models applied to hypoxia, vasculature and fluorescent reporters. Nat Protoc. 2011; 6(9):1355–66. Epub 2011/09/03. https://doi.org/10.1038/nprot.2011.349 PMID: 21866198

29. Trentham DE, Dynesius RA, David JR. Passive transfer by cells of type II collagen-induced arthritis in rats. J Clin Invest. 1978; 62(2):359–66. Epub 1978/08/01. https://doi.org/10.1172/JCI109136 PMID: 27553

30. Takai Y, Seki N, Senoh H, Yokota T, Lee F, Hamaoka T, et al. Enhanced production of interleukin-6 in mice with type II collagen-induced arthritis. Arthritis Rheum. 1989; 32(5):594–600. Epub 1989/05/01. PMID: 2785799

31. McEver RP, Cummings RD. Perspectives series: cell adhesion in vascular biology. Role of PSGL-1 binding to selectins in leukocyte recruitment. J Clin Invest. 1997; 100(3):485–91. Epub 1997/08/01. https://doi.org/10.1172/JCI119556 PMID: 9293993

32. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat Rev Immunol. 2007; 7(9):678–89. Epub 2007/08/25. https://doi.org/10.1038/nri2156 PMID: 17717539

33. Sissi C, Naggi A, Torri G, Palumbo M. Modulation of antithrombin-protease interactions by semisynthetic low-molecular-weight heparins with different sulfation patterns. Semin Thromb Hemost. 2003; 29(6):661–70. Epub 2004/01/14. https://doi.org/10.1055/s-2004-816533 PMID: 14719183

34. Thourani VH, Brar SS, Kennedy TP, Thornton LR, Watts JA, Ronson RS, et al. Nonanticoagulant heparin inhibits NF-kappaB activation and attenuates myocardial reperfusion injury. Am J Physiol Heart Circ Physiol. 2000; 278(6):H2084–93. Epub 2000/06/14. PMID: 10943908

35. Ashikari-Hada S, Habuchi H, Kariya Y, Itoh N, Reddi AH, Kimata K. Characterization of growth factor-binding structures in heparin/heparan sulfate using an octasaccharide library. J Biol Chem. 2004; 279(13):12346–54. Epub 2004/01/07. https://doi.org/10.1074/jbc.M310927200 PMID: 14707131

36. Rao NV, Argyle B, Xu X, Reynolds PR, Walenga JM, Prechel M, et al. Low anticoagulant heparin targets multiple sites of inflammation, suppresses heparin-induced thrombocytopenia, and inhibits interaction of RAGE with its ligands. Am J Physiol Cell Physiol. 2010; 299(1):C97–110. Epub 2010/04/08. https://doi.org/10.1152/ajpcell.00009.2010 PMID: 20375277
37. Fryer A, Huang YC, Rao G, Jacoby D, Mancilla E, Whorton R, et al. Selective O-desulfation produces nonanticoagulant heparin that retains pharmacological activity in the lung. J Pharmacol Exp Ther. 1997; 282(1):208–19. Epub 1997/07/01. PMID: 9223556

38. Vannucchi S, Pasquali F, Chiarugi V, Ruggiero M. Internalization and metabolism of endogenous heparin by cultured endothelial cells. Biochem Biophys Res Commun. 1986; 140(1):294–301. Epub 1986/10/15. PMID: 3778451

39. Barzu T, van Rijn JL, Petitou M, Tobelem G, Caen JP. Heparin degradation in the endothelial cells. Thromb Res. 1987; 47(5):601–9. Epub 1987/09/01. PMID: 3672438

40. Young E, Venner T, Ribau J, Shaughnessy S, Hirsh J, Podor TJ. The binding of unfractionated heparin and low molecular weight heparin to thrombin-activated human endothelial cells. Thromb Res. 1999; 96(5):373–81. Epub 1999/12/22. PMID: 10605952

41. von Andrian UH, Mackay CR. T-cell function and migration. Two sides of the same coin. N Engl J Med. 2000; 343(14):1020–34. Epub 2000/10/06. https://doi.org/10.1056/NEJM200010053431407 PMID: 11018170

42. Mueller SN, Hosiawa-Meagher KA, Konieczny BT, Sullivan BM, Bachmann MF, Locksley RM, et al. Regulation of homeostatic chemokine expression and cell trafficking during immune responses. Science. 2007; 317(5838):670–4. Epub 2007/08/04. https://doi.org/10.1126/science.1144830 PMID: 17673664

43. Christopherson KW 2nd, Campbell JJ, Travers JB, Hromas RA. Low-molecular-weight heparins inhibit CCL21-induced T cell adhesion and migration. J Pharmacol Exp Ther. 2002; 302(1):290–5. Epub 2002/06/18. PMID: 12065729

44. Dyer DP, Salanga CL, Volkman BF, Kawamura T, Handel TM. The dependence of chemokine-glycosaminoglycan interactions on chemokine oligomerization. Glycobiology. 2016; 26(3):312–26. Epub 2015/11/20. https://doi.org/10.1093/glycob/cwv100 PMID: 26582609

45. Buatois V, Fagete S, Magistrelli G, Chatel L, Fischer N, Kosco-Vilbois MH, et al. Pan-CC chemokine neutralization restricts splenocyte egress and reduces inflammation in a model of arthritis. J Immunol. 2010; 185(4):2544–54. Epub 2010/07/21. https://doi.org/10.4049/jimmunol.1000182 PMID: 20644170