이학석사학위논문

새로운 히스톤 디아세틸레이즈6 억제제인 CKD-H059의 류마티스 관절염 치료 효과

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a novel histone deacetylase 6 inhibitor,
in rheumatoid arthritis

2016년 2월

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장유진

장유진의 석사학위논문을 인준함.

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Abstract

Therapeutic effects of CKD-H059, a novel histone deacetylase 6 inhibitor, in rheumatoid arthritis

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Background: Epigenetic changes including histone modification may play a role in development of rheumatoid arthritis (RA). Histone deacetylase inhibitor (HDACi) could increase transcription of numerous genes by rendering chromatin state more accessible for transcription factor and RNA-polymerase, leading to anti-proliferative and anti-inflammatory effects.

Objective: This study was aimed to investigate the effects of CKD-H059, a novel HDAC-6 inhibitor, on peripheral blood mononuclear cells (PBMCs), regulatory

T (Treg) cells and fibroblast-like-synoviocytes (FLSs) of RA patients in vitro and on development of arthritis in animal model.

Methods: After 24 hours activation with LPS in the increasing concentrations of CKD-H059, TNF-α, IL-1β, IL-6 and IL-10 production of RA PBMC were assessed. Regulatory T cells (iTreg) were induced from naive CD4+ T cells of RA patients. CFSE labeled effector T cells (Teff) from healthy subjects were co-cultured with iTreg in the increasing concentrations of CKD-H059 and Teff proliferation was analyzed by flow cytometry. After activation with IL-1β in the increasing concentrations of CKD-H059, MMP-1, MMP-3, IL-6 and IL-8 production of RA-FLS were assessed. Cytoplasmic acetylation of α-tubulin in the activated RA-FLS was visualized by confocal microscopy. Rats with adjuvant-induced arthritis (AIA) were treated with oral CKD-H059 (3, 10, 30, 50, 100 mg/kg) once a day and the severity of arthritis was assessed on 9, 13, and 16 days.

Result: CKD-H059 decreased TNF-α and increased IL-10 of RA PBMCs without impact on cell viability. In the presence of CKD-H059, iTreg efficiently inhibited the proliferation of Teff in a dose dependent manner. CKD-H059 inhibited MMP-1, MMP-3, IL-6

and IL-8 and induced acetylation of α-tubulin in cytoskeleton with subsequent cell morphology change. In AIA rat, oral CKD-H059 was able to prevent the development of clinical arthritis in a dose-dependent manner.

Conclusion: The novel HDAC6 inhibitor CKD-H059 inhibits the inflammatory response in PBMCs and FLS of RA and restores Treg cell function. CKD-H059 ameliorates arthritis severity in animal model. Therefore, CKD-H059 might offer a novel treatment option for RA.

Key words : RA, HDAC inhibitor, CKD-H059, PBMC, Treg cells, FLS

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Contents

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by a chronic inflammation in the joints and surrounding connective tissues [1]. Inflammatory cytokines, such as tumor necrosis factor (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6), and fibroblast-like-synoviocytes (FLSs) that produce matrix metalloproteinases (MMPs) contribute to the joint destruction by pannus formation [2, 3]. Defective regulatory T (Treg) cells with decreased ability to suppress effector T (Teff) cells, have been reported to contribute to chronic inflammatory process during RA pathogenesis [4].

Epigenetic changes can be categorized into several groups: DNA methylation, histone modifications, nucleosome positioning and microRNA [5]. Epigenetic modification by histone acetyltransferases (HATs) and histone deacetylases (HDACs) are crucial in regulating gene expression and cell differentiation and function [6]. Accordingly, altered epigenetic modifications have been reported in various diseases including cancer and autoimmune disease such as RA and systemic lupus erythematosus (SLE) [5]. Histone acetyltransferase (HAT) activity leads to histone acetylation and

increases the accessibility of gene promoters for transcription, while histone deacetylases (HDACs) counteract the activity of HATs and so decreased the gene transcription [7]. Thus, HDAC inhibitors promote transcription of numerous genes by rendering chromatins more accessible for transcription factors [8]. In addition, other cellular protein beyond histones are substrate to the HDACs [5]. Taken together, HDAC inhibitor may ultimately exert anti-proliferative and anti-inflammatory effects [9].

Previous studies have shown that HDAC inhibitors have therapeutic effects in RA [10]. In contrast to pan-HDAC inhibitors, which suppress multiple HDAC isoforms, selective inhibitors targeting specific HDAC isoforms might be of particular interest as a therapeutic agent for autoimmune disease to reduce side effects such as fatigue, diarrhea, nausea, and neutropenia [11]. Among the HDAC inhibitors, HDAC6 inhibitors have been reported to be effective in treatment of RA with a tolerable safety profile [12].

This study aimed to investigate the therapeutic effects of CKD-H059, a novel HDAC6 inhibitor, as a potential drug candidate to treatment RA. Here, the effects of CKD-H059 on regulatory T (Treg) cells and fibroblast-like-synoviocytes (FLSs) of RA patients in

vitro as well as on the experimental arthritis in adjuvant-induced arthritis (AIA) model [13, 14] were investigated.

Materials and methods

Animal

Lewis rats (female, 5 weeks old) were purchased from Central Lab Animal, Inc (Seoul, Korea). Animals were provided with standard diet (Central Lab Animal, Inc.) and water ad libitum and housed in controlled environment with temperature (22 ± 2 ° C), humidity (44-56%) and 12 hours light-dark cycle.

Induction of experimental arthritis

Complete Freund' s adjuvant (Chondrex, Seattle, WA) was resuspended vigorously. A 100 μl of mixture was injected subcutaneously into the tail base of rats. Animals were randomized to 6 groups (vehicle, n = 7; CKD-H059 3 mg/kg, n = 8; CKD-H059 10 mg/kg, n = 8; CKD-H059 30 mg/kg, n = 8; CKD-H059 50 mg/kg, n = 8; CKD-H059 100 mg/kg, n = 8). Each group was treated with vehicle or oral CKD-H059 once a day from day -1 to day 16. The severity of arthritis was assessed on days 9, 13, and 16 after adjuvant injection.

Arthritis assessment

The severity of arthritis was evaluated by scoring arthritis of each joint (digits, metatarsal bone, tarsal

bone) as follows: 0, no swelling or erythema; 1, slight swelling and/or erythema; 2, low to moderate edema; 3, pronounced edema with limited joint usage; 4, excess edema with joint rigidity. The clinical scores of four joints were summed up a total score in each animal.

Cell culture

PBMCs were isolated from peripheral blood of RA patients by Ficoll-Hypaque density centrifugation and resuspended in RPMI-1640 containing 1% fetal bovine serum and 1% penicillin/streptomycin. Cells were pre-incubated for one hour with medium alone, tubastatin A (1 μM) or H059 (1, 2, 5 μM), then were stimulated for 24 hours with lipopolysaccharide (LPS) (SIGMA, St.Louis, MO) at 100 ng/ml.

CD4+ CD25- T cells were purified from RA PBMCs by negative selection using CD4+ T Cell Biotin-Antibody Cocktail (Miltenyi Biotec, Auburn, CA). Treg cells were generated from CD4+ CD25- T cells in the presence of anti-CD3 antibody (eBioscience, San Diego, CA), anti-CD28 antibody (BD Pharmingen, San Diego, CA), IL-2 (PEPROTECH, Rocky Hill, NJ), TGF-β (PEPROTECH) and vitamin D3 (SIGMA) [15].

FLSs were isolated from synovial biopsies of RA

patients and cultured in DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin. Cells from passages between three and seven were used for experiments. FLSs were pre-incubated for one hour with medium alone, tubastatin A (1, 5 μM) or CKD-H059 (1, 2, 5 μM), then were stimulated for 6 hours or 24 hours with IL-1β (R&D, Minneapolis, MN) at 10 ng/ml.

Reagent

CKD-H059 was provided by Chong Kun Dang Pharmaceutical Corp (Seoul, Korea). Tubastatin A (SIGMA) were used as a positive control.

Measurement of cell viability

Viability of PBMC and FLS was evaluated after two hours incubation with CCK-8 (DOJINDO, Kumamoto, Japan) and optical density was read at 450 nm.

Measurement of cytokines in cell culture supernatants

RA PBMCs were treated with HDAC6 inhibitors and then stimulated with LPS. RA FLSs were treated with HDAC6 inhibitors and then stimulated with IL-1β. After 24 hours, cell culture supernatants were collected, and concentration of TNF-α, IL-1β, IL-6

and IL-10 in PBMCs and MMP-1, MMP-3, IL-6 and IL-8 in FLSs were measured using enzyme-linked immunosorbent assay (ELISA).

Suppression of effector T (Teff) cell proliferation

CD4+ CD25- T cells were purified from healthy PBMCs by negative selection using CD4+ T Cell Biotin-Antibody Cocktail (Miltenyi Biotec) and were subsequently labeled with 5 mM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Life Technologies, Eugene, OR) for 10 minutes. The induced Treg (iTreg) cells and CFSE-labeled Teff cells were co-cultured at ratio of 0:1, 0.3:1 and 1:1 in the presence of Dynabeads Human T-Activator CD3/CD28 (Life Technologies, Oslo, AS) for 72 hours. Thereafter, the cells were harvested and proliferation of Teff was determined using flow cytometry.

Protein extraction and immunoblotting

In order to determine acetylation of α-tubulin, FLSs were pretreated with HDAC6 inhibitors and then were stimulated with IL-1β. Cells were lysed by M-PER (Thermo, Rockford, IL) and the lysates were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The blots were incubated with mouse

anti-acetylated-tubulin antibody (1:1000) (SIGMA) followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (1:5000) (Jackson ImmunoResearch, West Grove, PA).

The blots were bound with rabbit anti-α-tubulin antibody (1:1000) (SIGMA) followed by incubation with HRP-conjugated goat anti-rabbit antibody (1:5000) (Abcam, Cambridge, UK) and exposure in X-ray film.

Immonofluorescence staining

Stimulated FLSs, were stained for visualization of acetylation of tubulin. The cells were permeabilized by 4% Triton X-100 (SIGMA) and stained with mouse anti-acetylated-tubulin antibody (1:1000) (SIGMA) followed by with Alexa 488-conjugated donkey anti-mouse antibody (1:5000) (Life Technologies). The cells were stained with rabbit anti-α-tubulin antibody (1:1000) (SIGMA) followed by stain with Alexa 594-conjugated goat anti-rabbit antibody (1:5000) (Life Technologies). Finally, the stained cells were taken by confocal microscopy.

Statistical analysis

Data are presented as the mean ± SEM. Statistical analyses were performed using paired t-tests in Prism

software (GraphPad). P values less than 0.05 were considered statically significant.

Results

CKD-H059 suppresses production of pro-inflammatory cytokines

We assessed the ability of CKD-H059 to suppress the production of pro-inflammatory cytokines by PBMCs following LPS stimulation. PBMCs from RA were pre-treated with increasing concentrations of CKD-H059 or tubastatin A for 1 hour and then were stimulated with LPS. Cell toxicity of CKD-H059 was evaluated using the CCK-8 assay. CKD-H059 (1 μM ~ 5 μM) had no significant effects on the viability of RA PBMCs (Figure 1). CKD-H059 reduced the production of TNF-α in a dose-dependent manner. As compared with cells treated with LPS only (3376 $\pm$ 393.9 pg/ml), CKD-H059 inhibited TNF-α production at a concentration of 1 μM (2511 $\pm$ 342.4 pg/ml, p = 0.002), 2 μM (1609 $\pm$ 166.7 pg/ml, p = 0.002) and 5 μM (500.1 $\pm$ 52.47 pg/ml, p = 0.002) (Figure 2A).

However, the production of other pro-inflammatory cytokines such as IL-1β and IL-6 was not significantly inhibited by CKD-H059 (Figure 2B, 2C). In order to evaluate the effect of CKD-H059 on production of anti-inflammatory cytokines, the production of IL-10 was measured. CKD-H059 at a

concentration of 1 μM (165.0 ± 23.58 pg/ml, p = 0.002) and 2 μM (127.7 ± 14.20 pg/ml, p = 0.002) significantly increased production of IL-10 as compared to cells treated with LPS only (79.80 ± 13.35 pg/ml) (Figure 2D).

CKD-H059 restores Treg function from RA patients

iTreg cells from RA patients have been reported to be dysfunctional. To test whether CKD-H059 improves Treg function from RA patients, the generated iTregs were co-cultured with CFSE-labeled Teff cells from healthy PBMCs in the increasing concentrations of CKD-H059. In the absence of CKD-H059, as the ratio of iTreg to Teff increased, proliferation of Teff cell decreased (Figure 3). In the presence of CKD-H059, the level of Teff cell proliferation decreased further. Interestingly, CKD-H059 was able to inhibit proliferation of the Teff cell even in the absence of iTregs. The lowest proliferation of Teff was achieved when Teff cells were co-cultured at the ratio of 1:1 in the presence of CKD-H059 at 2 μM.

CKD-H059 suppresses fibroblast-like synoviocytes

Next, we investigated whether CKD-H059 had an impact on the immune response of RA-FLSs upon IL-1 β stimulation. After RA-FLSs had been treated with

increasing concentrations of CKD-H059 or tubastatin A (as a positive control) for 1 hour, they were stimulated with IL-1β. CKD-H059 (1 μM and 5 μM) did not significantly influence the viability of RA-FLSs, as shown Figure 4.

In RA, MMP is markedly secreted and contribute to the destruction of joint tissue. RA-FLS were treated with IL-1β in the presence of CKD-H059 for 24 hours and the production of MMP-1 and MMP-3 were measured. Compared with cells stimulated with IL-1β only (31994 ± 15312 pg/ml), CKD-H059 inhibited MMP-1 at concentration of 1 μM (27603 ± 14292 pg/ml, p = 0.0313) and 5 μM (18347 ± 10487 pg/ml, p = 0.0313) (Figure 5A). And Compared with cells stimulated with IL-1β only (80037 ± 14248 pg/ml), CKD-H059 inhibited MMP-3 production at concentration of 1 μM (61394 ± 16325 pg/ml, p = 0.0313) and 5 μM (35284 ± 13598 pg/ml, p = 0.0313) (Figure 5B).

The effect of CKD-H059 on production of cytokine, IL-6 and IL-8, in RA FLSs was examined using ELISA. Cells treated with IL-1β showed a notable increase in IL-6 and IL-8 production. IL-6 production by FLSs was inhibited following treatment with CKD-H059 (12934 ± 2254 vs 8865 ± 1055, p = 0.0313) (Figure

6A). IL-8 production was significantly reduced when FLSs were treated with CKD-H059 (19311 ± 1068 vs 11215 ± 360.7, p = 0.0313) (Figure 6B).

CKD-H059 changes cytoskeleton

Among various cellular proteins, tubulins, which constitute the major cytoskeleton microtubules, are substrate to acetylation [16]. CKD-H059 as a HDAC6 inhibitor decreases the deacetylation and so should keep the cellular proteins more in acetylated state. The acetylated tubulins might lose their physiological function that is translated into morphological changes of cytoskeleton [17, 18]. RA-FLS were treated with IL-1β in the presence of CKD-H059 for 24 hours and the acetylated tubulins were analysed using Western blot and confocal microscopy. As compared to media or IL-1β-only conditions, cells treated with CKD-H059 had more acetylated tubulin. This was associated with cell shape; spindle-like cells became rounder, occupying more surface area (Figure 7).

CKD-H059 ameliorates experimental arthritis in rat

The efficacy of CKD-H059 on inflammatory arthritis was evaluated in AIA model. The rats were treated with oral CKD-H059 at 3, 10, 30, 50, and 100 mg/kg. Clinical scores reduced by 14.1% ($p > 0.05$), 32.4% (p

> 0.05), 73.2% (p < 0.01), 85.9% (p < 0.01) and 100.0% (p < 0.01) from vehicle group respectively on day 16 (Figure 8), suggesting that CKD-H059 might have therapeutic effect in the treatment of inflammatory arthritis.

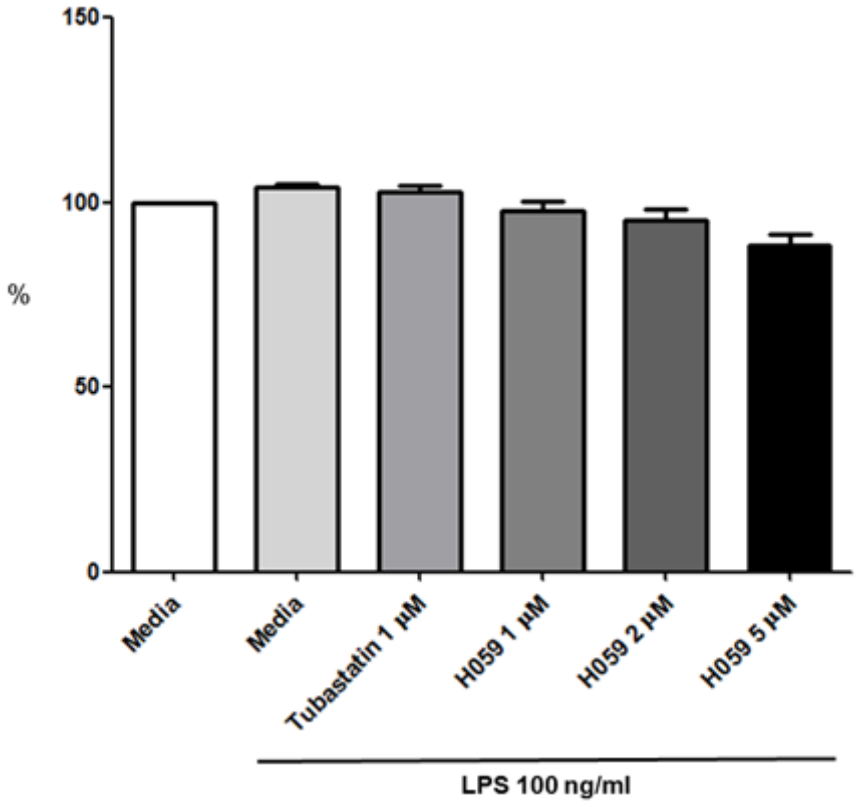

Figure 1. CKD-H059 does not affect viability of PBMCs. PBMCs were isolated from RA patients and were treated with increasing concentrations of CKD-H059. Cell toxicity was evaluated using CCK-8 assay. CKD-H059 (1 μM ~ 5 μM) had no significant effect on the viability of RA PBMCs (n=5) with LPS 100 ng/ml. Data represent mean value ± SEM.

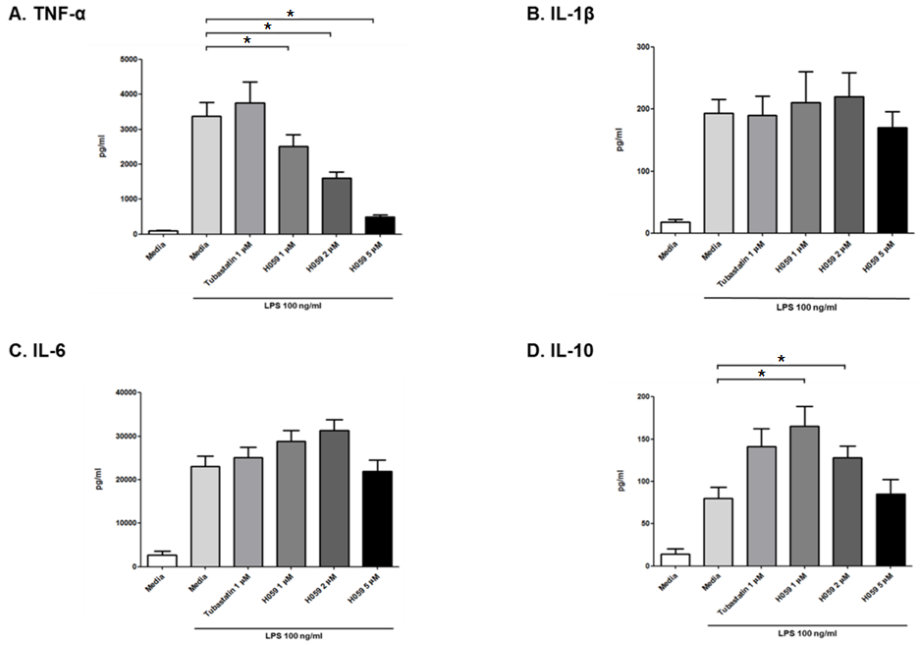

Figure 2. CKD-H059 significantly suppresses TNF-α and enhances the IL-10 production in RA PBMCs. (A) PBMCs from RA patients (n=5) were stimulated with LPS in the increasing concentrations of CKD-H059 and the production TNF-α was measured. CKD-H059 (1 μM ~ 5 μM) reduced the TNF-α production by PBMCs dose-dependently (*p < 0.01). (B) The production of IL-1β was not significantly suppressed by CKD-H059. (C) The production of IL-6 was not significantly suppressed by CKD-H059. (D) CKD-H059 (1 μM and 2 μM) enhanced the production of IL-10 of RA PBMCs (*p < 0.01). All data represent mean value ± SEM.

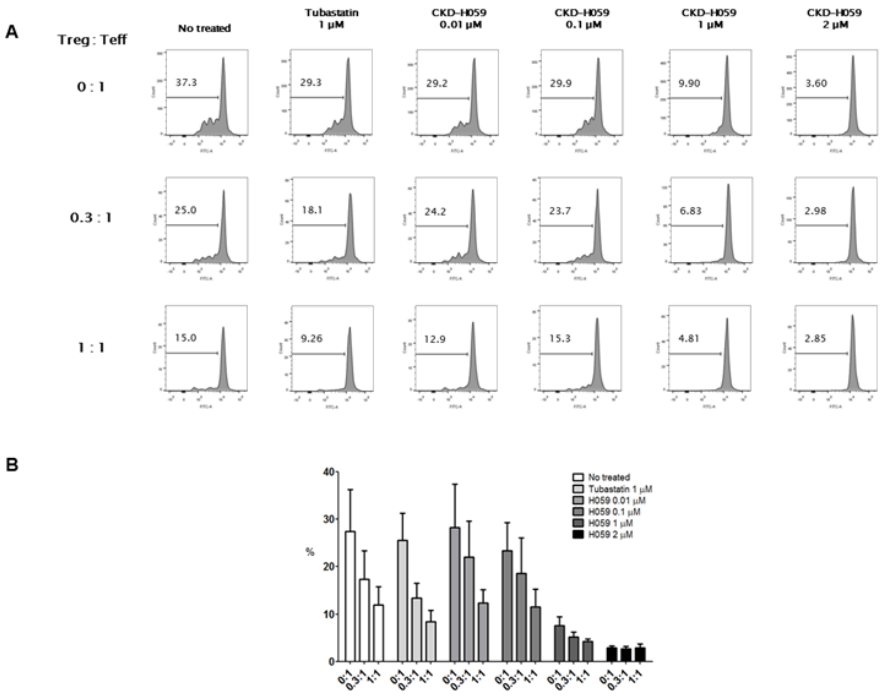

Figure 3. CKD-H059 restores Treg function. (A) CKD-H059 augments the inhibitory effect of Tregs on proliferation of CFSE-labeled Teff cells from 3 independent experiments (representative data). (B) Mean percent suppression of Teff cells proliferation by Treg cells in the presence of CKD-H059 (n=3). Data represent mean value ± SEM.

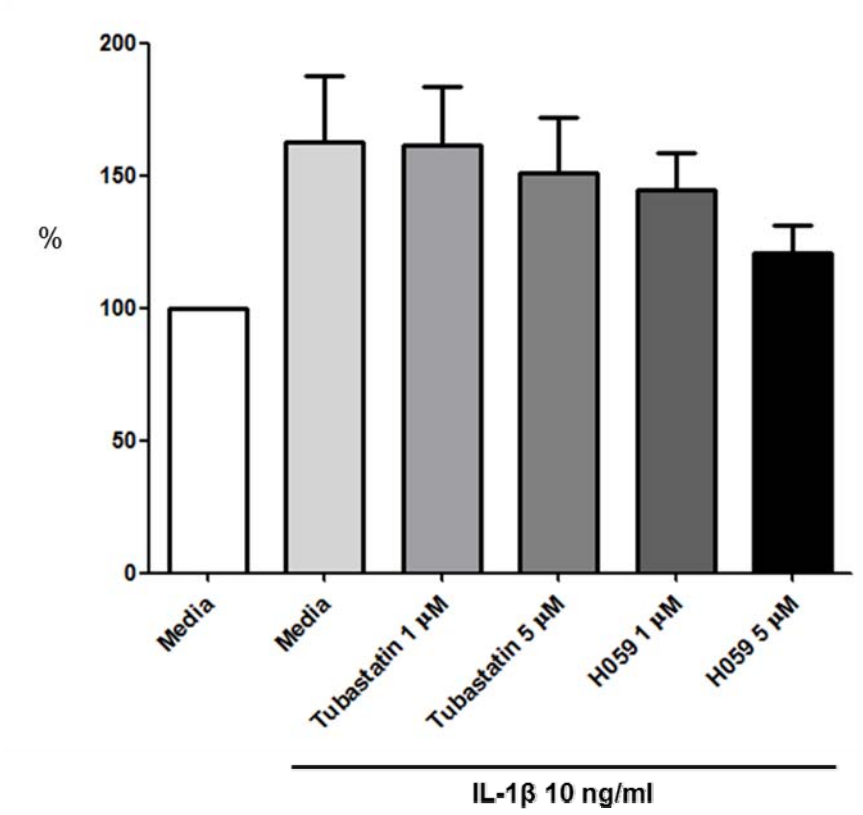

Figure 4. CKD-H059 does not affect viability of RA FLSs in the presence of IL-1β stimulation. Based on the CCK-8 assay, CKD-H059 (1 μM and 5 μM) had no significant effect on the viability of RA FLSs (n=3) with IL-1β 10 ng/ml. Data represent mean value ± SEM.

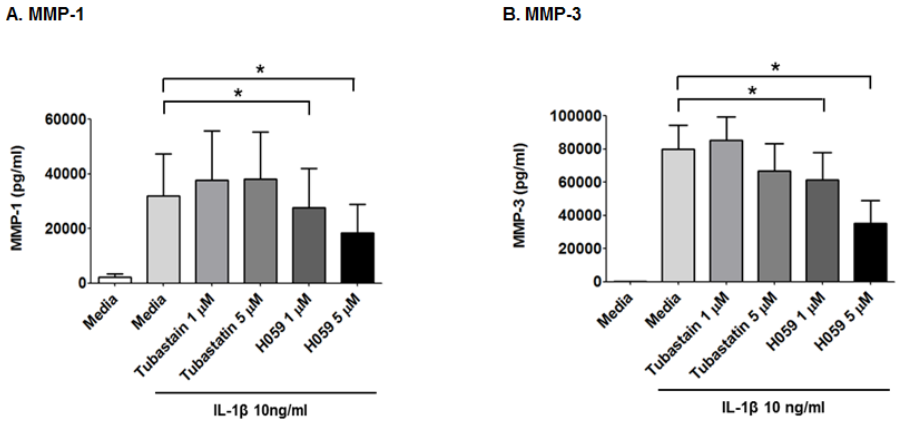

Figure 5. CKD-H059 significantly suppresses secretion of MMP-1 and MMP-3 in RA FLSs stimulated with IL-1β. CKD-H059 (1 μM and 5 μM) reduced the production of MMP-1 (A) and MMP-3 (B) of RA FLSs (n=3) dose-dependently (*$p < 0.05$ respectively). Data represent mean value ± SEM.

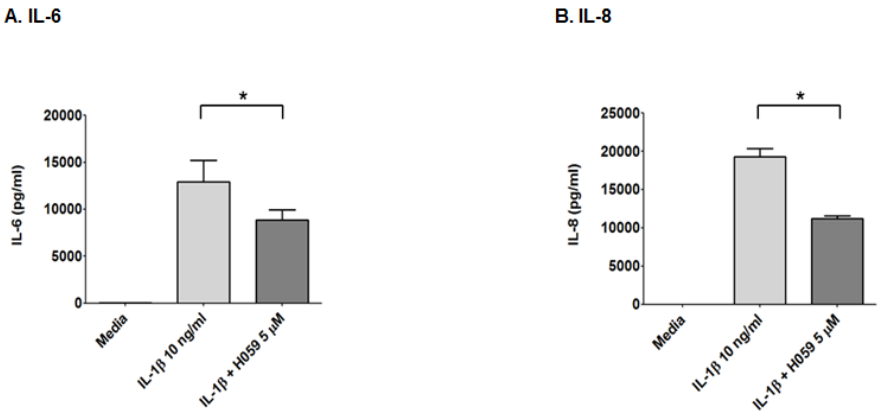

Figure 6. CKD-H059 significantly suppresses IL-6 and IL-8 secretion in RA FLSs stimulated with IL-1β. CKD-H059 (5 μM) reduced the production of IL-6 (A) and IL-8 (B) of RA FLSs (n=3) (*$p < 0.05$ respectively). Data represent mean value ± SEM.

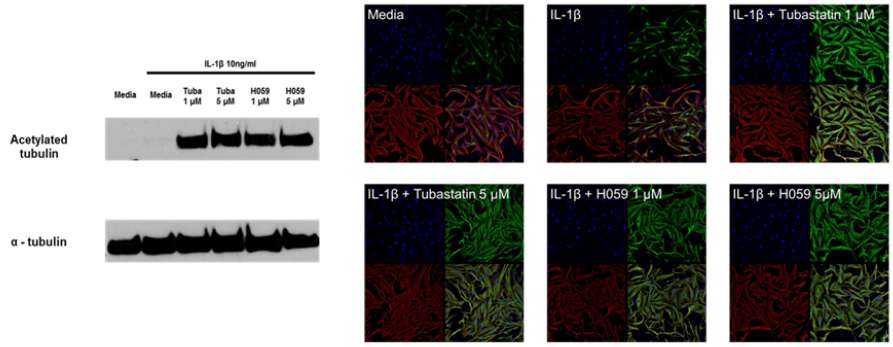

Figure 7. CKD-H059 acetylates α-tubulin in IL-1β -stimulated RA FLS. Acetylated tubulin increased in the presence of a tubastatin A or CKD-H059 condition (1 μM and 5 μM) in FLS following stimulation with IL-1β 10 ng/ml (n=3). By comparison, α-tubulin expression remained constant (blue, nucleus; green, acetylated tubulin; red, α-tubulin).

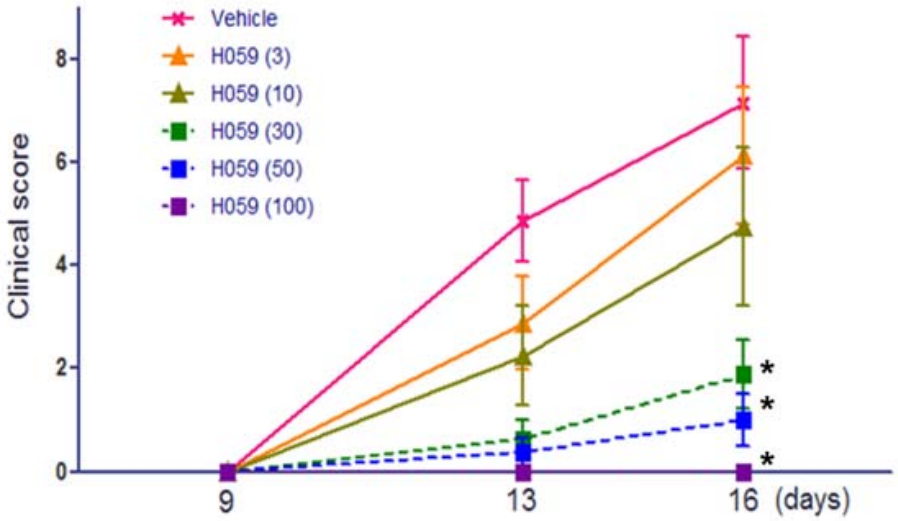

Figure 8. CKD-H059 ameliorates experimental arthritis in AIA rat model. Arthritis was induced as described in Methods and rats were treated with increasing concentrations of CKD-H059. Rats treated with CKD-H059 (30, 50, 100 mg/kg) showed statistically significant reduction in clinical score (*p < 0.01). Data represent mean value ± SEM.

Discussion

RA is an autoimmune disease of unknown etiology that manifests by chronic articular inflammation and extra-articular manifestation [19]. Dysfunction in both humoral and cellular immune responses results in chronic inflammation during the RA development, where the pro-inflammatory autoimmune response is not effectively regulated by anti-inflammatory mechanisms [19]. As a result, continuous activation of immune system has pleiotropic effects and transforms synoviocytes into fibroblast-like cells that form pannus and mediate joint destruction [20]. In this regard, a drug that enhances the regulatory mechanism might be of a particular interest in RA treatment.

Differentiation and function of cells are controlled by their gene expression that are regulated by epigenetic modification including DNA methylation, histone modification, nucleosome positioning and microRNA [5, 21]. Therefore, regulation of enzymes involved in histone acetylation and deacetylation may contribute to cell differentiation and function.

HDAC6 is localized in the cytoplasm unlike other HDACs, where it associates with non-histone substrates, such as HSP90 and α-tubulin [22]. It is

reported that tubastatin, HDAC6 selective inhibitor, induces α-tubulin hyperacetylation and has anti-inflammatory effects [23, 24]. Our findings demonstrated that CKD-H059, a novel HDAC6 inhibitor, influences the acetylation state of tubulin. In addition, it has pleiotropic effects on both immune cells and FLS which constitute the main cellular infiltrates in joints of RA. CKD-H059 significantly decreased pro-inflammatory cytokine TNF-α and increased anti-inflammatory cytokine IL-10 in RA PBMC. Since CKD-H059 inhibited TNF-α but not IL-1β, it is possible that CKD-H059 inhibits the TNF-α-specific intracellular pathway but not the inflammasome activation.

Treg cells contribute to maintaining self-tolerance by down-regulating immune response [25]. Abnormality of tolerance can leads to the development of various autoimmune diseases including RA [26]. CKD-H059 improved or restored the decreased Treg activity of RA patients. Moreover, CKD-H059 directly suppresses the proliferation of Teff cells. Taken together, CKD-059 suppresses immune response by directly inhibiting the proliferation of effector cells and by augmenting the function of regulatory T cells. Restoration of the defective Treg function is crucial for self-tolerance

and taming aggressive autoimmune cells from self-destruction.

In RA, inflammatory environment transforms the normal resident synoviocytes into aggressive FLSs that produce IL-6, IL-8 and the extracellular matrix proteinases including MMP-1 and MMP-3 [27, 28]. Since CKD-H059 markedly inhibited the secretion of MMP-1 and MMP-3, it might prevent also joint destruction mediated by aggressive FLS. Also, CKD-H059 significantly inhibited the production of IL-6 and IL-8 in FLSs. Strikingly, CKD-H059 keeps cytosolic tubulin acetylated within FLSs. This may be associated with a clear change in cell morphology and migration [18, 29].

Exact mechanisms of suppression of experimental arthritis by CKD-H059 remains to be elucidated. CKD-H059 mediated histone acetylation may render the promoters for proteins involved in inflammatory signaling such as NF-κB and JAK-STAT signaling less accessible for transcription factors [30, 31]. Since IL-1β production was not influenced by CKD-H059, inflammasome pathway might not be influenced by CKD-H509 [32].

It is reported that Treg cells from RA patients treated with methotrexate (MTX), a disease-modifying

anti-rheumatic drug (DMARDs), restores suppressive function through increased expression of FoxP3 and CTLA-4 in Treg cells [33]. Therefore, the combination of CKD-H059 and MTX may have a synergetic therapeutic effect in RA.

In conclusion, CKD-H059, a novel HDAC6 inhibitor, promotes anti-inflammatory response and ameliorates the experimental arthritis. CKD-H059 might provide a novel treatment option in the treatment of RA.

References

1. Feldmann M, Brennan FM, Maini RN: Rheumatoid arthritis. Cell, 1996, 85(3):307-310.

2. Brennan FM, McInnes IB: Evidence that cytokines play a role in rheumatoid arthritis. J Clin Invest, 2008, 118(11):3537.

3. Mor A, Abramson SB, Pillinger MH: The fibroblast-like synovial cell in rheumatoid arthritis: a key player in inflammation and joint destruction. Clin Immunol, 2005, 115(2):118-128.

4. Lawson CA, Brown AK, Bejarano V, Douglas SH, Burgoyne CH, Greenstein AS, Boylston AW, Emery P, Ponchel F, Isaacs JD: Early rheumatoid arthritis is associated with a deficit in the CD4+CD25high regulatory T cell population in peripheral blood. Rheumatology (Oxford), 2006, 45(10):1210-1217.

5. Portela A, Esteller M: Epigenetic modifications and human disease. Nat Biotech, 2010, 28(10):1057-1068.

6. Peserico A, Simone C: Physical and functional HAT/HDAC interplay regulates protein acetylation balance. Biomed Res Int, 2010, 2011.

7. Urnov FD, Wolffe AP: Chromatin remodeling and transcriptional activation: the cast (in order of appearance). Oncogene, 2001, 20(24):2991-3006.

8. Marks PA, Richon VM, Miller T, Kelly WK: Histone deacetylase inhibitors. Adv Cancer Res, 2004, 91:137-168.
9. Blanchard F, Chipoy C: Histone deacetylase inhibitors: new drugs for the treatment of inflammatory diseases? Drug Discov. Today, 2005, 10(3):197-204.
10. Grabiec AM, Krausz S, de Jager W, Burakowski T, Groot D, Sanders ME, Prakken BJ, Maslinski W, Eldering E, Tak PP: Histone deacetylase inhibitors suppress inflammatory activation of rheumatoid arthritis patient synovial macrophages and tissue. J immunol, 2010, 184(5):2718-2728.
11. Regna NL, Reilly CM: Isoform-selective HDAC inhibition in autoimmune disease. J Clin Cell Immunol, 2014, 2014.
12. Vishwakarma S, Iyer LR, Muley M, Singh PK, Shastry A, Saxena A, Kulathingal J, Vijaykanth G, Raghul J, Rajesh N et al: Tubastatin, a selective histone deacetylase 6 inhibitor shows anti-inflammatory and anti-rheumatic effects. Int Immunopharmacol, 2013, 16(1):72-78.
13. Refaat R, Salama M, Meguid EA, El Sarha A, Gowayed M: Evaluation of the effect of losartan and methotrexate combined therapy in adjuvant-induced arthritis in rats. Eur J Pharmacol, 698.1 (2013):

421-428.
14. Woods JM, Katschke KJ, Volin MV, Ruth JH, Woodruff DC, Amin MA, Connors MA, Kurata H, Arai K, Haines GK, Kumar P, Koch AE: IL-4 adenoviral gene therapy reduces inflammation, proinflammatory cytokines, vascularization, and bony destruction in rat adjuvant-induced arthritis. J immunol, 166.2 (2001): 1214-1222.
15. Jeffery LE, Burke F, Mura M, Zheng Y, Qureshi OS, Hewison M, Walker LS, Lammas DA, Raza K, Sansom DM: 1, 25-Dihydroxyvitamin D3 and IL-2 combine to inhibit T cell production of inflammatory cytokines and promote development of regulatory T cells expressing CTLA-4 and FoxP3. J immunol, 2009, 183(9):5458-5467.
16. Palazzo A, Ackerman B, Gundersen GG: Cell biology (Communication arising): Tubulin acetylation and cell motility. Nature, 2003, 421(6920):230-230.
17. Valenzuela-Fernandez A, Cabrero JR, Serrador JM, Sánchez-Madrid F: HDAC6: a key regulator of cytoskeleton, cell migration and cell-cell interactions. Trends Cell Biol, 2008, 18(6):291-297.
18. Jung H-Y, Jung JS, Whang YM, Kim YH: RASSF1A suppresses cell migration through inactivation of HDAC6 and increase of acetylated α-tubulin. Cancer

Res Treat, 2013, 45(2):134.
19. Burmester GR, Feist E, Dorner T: Emerging cell and cytokine targets in rheumatoid arthritis. Nat Rev Rheumatol, 2014, 10(2):77-88.
20. Bartok B, Firestein GS: Fibroblast-like synoviocytes: key effector cells in rheumatoid arthritis. Immunol Rev, 2010, 233(1):233-255.
21. Rice JC, Allis CD: Histone methylation versus histone acetylation: new insights into epigenetic regulation. Curr Opin Cell Biol, 2001, 13(3):263-273.
22. Xu W, Parmigiani R, Marks P: Histone deacetylase inhibitors: molecular mechanisms of action. Oncogene, 2007, 26(37):5541-5552.
23. Butler KV, Kalin J, Brochier C, Vistoli G, Langley B, Kozikowski AP: Rational design and simple chemistry yield a superior, neuroprotective HDAC6 inhibitor, tubastatin A. J Am Chem Soc, 2010, 132(31):10842-10846.
24. Vishwakarma S, Iyer LR, Muley M, Singh PK, Shastry A, Saxena A, Kulathingal J, Vijaykanth G, Raghul J, Rajesh N: Tubastatin, a selective histone deacetylase 6 inhibitor shows anti-inflammatory and anti-rheumatic effects. Int Immunopharmacol, 2013, 16(1):72-78.
25. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda

M: Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J immunol, 1995, 155(3):1151-1164.
26. Sakaguchi S: Regulatory T cells: key controllers of immunologic self-tolerance. Cell, 2000, 101(5):455-458.
27. Tolboom T, Pieterman E, Van Der Laan W, Toes R, Huidekoper A, Nelissen R, Breedveld F, Huizinga T: Invasive properties of fibroblast-like synoviocytes: correlation with growth characteristics and expression of MMP-1, MMP-3, and MMP-10. Ann Rheum Dis, 2002, 61(11):975-980.
28. Firestein GS: Invasive fibroblast-like synoviocytes in rheumatoid arthritis. Passive responders or transformed aggressors? Arthritis Rheum, 1996, 39(11):1781-1790.
29. Dallol A, Agathanggelou A, Fenton SL, Ahmed-Choudhury J, Hesson L, Vos MD, Clark GJ, Downward J, Maher ER, Latif F: RASSF1A interacts with microtubule-associated proteins and modulates microtubule dynamics. Cancer Res, 2004, 64(12):4112-4116.
30. Yu Z, Zhang W, Kone BC: Histone deacetylases

augment cytokine induction of the iNOS gene. J Am Soc Nephrol, 2002, 13(8):2009-2017.
31. Klampfer L, Huang J, Swaby L-A, Augenlicht L: Requirement of histone deacetylase activity for signaling by STAT1. J Biol Chem, 2004, 279(29):30358-30368.
32. Latz E: The inflammasomes: mechanisms of activation and function. Curr Opin Immunol, 2010, 22(1):28-33.
33. Cribbs AP, Kennedy A, Penn H, Amjadi P, Green P, Read JE, Brennan F, Gregory B, Williams RO: Methotrexate restores regulatory T cell function through demethylation of the FoxP3 upstream enhancer in patients with rheumatoid arthritis. Arthritis Rheum, 2015, 67(5):1182-1192.

국문 초록

새로운 히스톤 디아세틸레이즈6 억제제인 CKD-H059의 류마티스 관절염 치료 효과

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장유진

배경: 히스톤 변형을 포함한 후성유전학적 변화는 류마티스 관절염의 발생에 중요한 역할을 할 수 있다. 히스톤 디아세틸레이즈 억제제는 전사인자와 RNA 중합효소가 염색질에 접근하기 용이하게 만들어 다양한 유전자의 전사를 증가시킴으로써 항증식 효과와 항염증 효과를 유도한다고 알려져 있다.

목표: 새로운 히스톤 디아세틸레이즈6 억제제인 CKD-H059가 류마티스 관절염 환자의 말초혈액단핵세포, 조절 T세포와 활막세포에 미치는 영향을 *in vitro* 로 조사하고 관절염동물 모델에서의 효과를 평가하고자 하였다.

실험방법: 류마티스 관절염 환자의 말초혈액단핵세포에 CKD-H059를 처리하고 LPS 자극을 준 후 TNF-α, IL-1β, IL-6, IL-10을 측정하였다. 또 류마티스 관절염환자의 CD4+ T 세포에서 유도한 조절 T 세포와 CFSE로 염색한 정상인의 T 세포를 함께 배양하여 CKD-H059를 처리한 후 유세포 분석기를 이용하여 T 세포의 증식 억제정도를 측정하였다. 또한 류마티스 관절염

환자의 활막세포에 CKD-H059를 처리하고 LPS 자극을 준 후 MMP-1, MMP-3, IL-6, IL-8을 측정하고 아세틸화 된 튜불린을 공초점현미경을 통해 확인하였다. 그리고 adjuvant-induced arthritis (AIA) 쥐 모델에서 CKD-H059 (3, 10, 30, 50, 100 mg/kg) 를 매일 경구 투여하고 9, 13, 16일에 관절염을 평가하였다.

실험결과: 말초혈액단핵세포에 CKD-H059를 처리하였을 때, 세포 생존에 영향없이 TNF-a가 유의하게 억제되고, IL-10이 유의하게 증가되었다 ($p < 0.01$). T 세포 억제실험 결과 CKD-H059에 의해 조절 T 세포의 기능이 회복되었다. 활막세포에서는 MMP-1와 MMP-3가 유의하게 억제되었고 ($p < 0.05$), IL-6와 IL-8이 유의하게 억제되었으며 ($p < 0.05$), 웨스턴 블롯과 공초점 현미경 결과 아세틸화 된 튜불린이 증가하는 것을 관찰하였다. AIA 모델에서 CKD-H059를 투여한 결과 대조군에 비해 유의하게 관절염 발생이 억제되었다 ($p < 0.01$).

결론: 새로운 히스톤 디아세틸레이즈6 억제제인 CKD-H059는 류마티스 관절염 환자의 말초혈액단핵세포와 활막세포에서 염증 반응을 억제시키고 조절 T 세포의 기능을 회복시키며, 동물모델에서 관절염을 억제시킴으로써 류마티스 관절염에 치료 효과가 있을 것으로 기대된다.

주요어 : 류마티스, 히스톤 디아세틸레이즈6 억제제, CKD-H059, 말초혈액단핵세포, 조절 T 세포, 활막세포

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