[Supplementary data to BMB Reports]

Title: Therapeutic effects of selective p300 histone acetyl-transferase inhibitor on liver fibrosis

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Running Title: Improvement of liver fibrosis by novel p300 inhibitor

Keywords: p300, histone acetyltransferase, liver fibrosis, inhibitor, A6

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Materials and Methods

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1. Experimental animals

All animal experiments were approved by Yonsei University College of Medicine Institutional Animal Care and Use Committee (IACUC No. 2019-0313) Eight-week-old male C57BL/6 mice were purchased from ORIENT BIO Inc. (Seongnam, Korea). The mice were maintained in specific-pathogen-free (SPF) and 12-hour light/dark cycle conditions. At the end of each time point, mice were weighed and then sacrificed under anesthesia induced by zoletil (50 mg/ml) and rumpun (23.32 mg/ml).

2. CD-HFD mouse model

The CD-HFD mouse group was fed a choline-deficient, L-amino acid-defined, high-fat diet (CD-HFD) with 60 kcal% fat with 0.1% methio-nine and no added choline (Research Diets Inc. NJ, USA) for 1-8 weeks. C646 (Selleck chemicals LLC. TX, USA) and A6 (synthesized from Dr. Hwang, S. Y. [66]) were dissolved in DMSO (Sigma, MO. USA), polyethylene glycol (Sigma. MO. USA), and PBS. Each chemical solution was administered 3 times/week for 8 weeks at a dose of 5mg/kg by intraperitoneal injection.

3. Thioacetamide model

Thioacetamide (TAA, Sigma-Aldrich, MO, USA) was dissolved in saline and administered daily at a dose of 200 mg/kg by intraperitoneal injection for 2 weeks. C646 and A6 were administered under the same conditions as listed above for the CD-HFD model.

4. Biochemical analyses

Blood samples were collected from the abdominal aorta of anesthetized mice, and whole blood was separated from the serum by centrifugation. Triglycerides (TG), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were measured with a biochemical analyzer (Dri-chem NX500, FUJIFILM, Tokyo, Japan).

5. RNA isolation and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted from fresh liver tissues and LX2 cell lines using the RNAiso Plus reagent (TaKaRa Bio, Otsu, Japan) according to the manufacturer's instructions. The RNA quantity and purity were determined using a NanoDrop 2000

spectrophotometer (Thermo Fisher Scientific, MA, USA). RNA samples were reverse-transcribed using Cell-script (CellSafe, Yongin, Korea) following the manufacturer's protocol. qRT-PCR analyses were performed using FastStart Universal SYBR Green Master (ROX) reagents (Roche, Basel, Switzerland) and ABI Prism 7700 sequence detection system (Applied Biosystems, CA, USA). Target gene expression levels were normalized by comparison to GAPDH expression levels. All reactions were performed in triplicate. The primer sets used for amplification are listed in Supplementary Table 1.

6. Immunoprecipitation assay

Cells were lysed in lysis buffer (50 mM Tris HCl pH 7.4, NaCl 150 mM, Triton X100 0.2%, NP-40 0.3%, EGTA 1 mM, EDTA 1 mM, Na3VO4 1 mM, NaF 1 mM, Xpert proteinase inhibitor cocktail (Gene depot. TX, USA)). Protein lysates were collected after centrifugation (12,000 rpm at 4˚C for 20 minutes). A 2% input sample was separated and the cell lysates were incubated with HA-magnetic beads (Sigma-Aldrich, MA, USA) for 2 hours at room temperature. After incubation, the lysate and bead mixture was placed on a magnetic rack and the supernatant was re-moved. The beads were washed with TBST for 15 min RT and then placed back on the magnetic rack. The supernatant was removed twice. Proteins were eluted in 1x SDS sample buffer (50% Glycerol, 20% SDS, 0.1% bromophenol blue sol., 0.5% 2-mercaptoethanol) after boiling for 10 minutes at 95˚C.

7. Western blot assay

Cells and tissue were lysed in lysis buffer (50 mM Tris HCl pH 7.4, NaCl 150 mM, Triton X100 0.2%, NP-40 0.3%, EGTA 1 mM, EDTA 1 mM, Na3VO4 1 mM, NaF 1 mM, Xpert proteinase inhibitor cocktail (Gene de-pot, TX, USA)). Protein lysates were collected after centrifugation at 12,000 rpm at 4˚C for 20 minutes and then boiled with SDS sample buffer (50% Glycerol, 20% SDS, 0.1% bromophenol blue sol., 0.5% 2-mercaptoethanol) at 95˚C for 5 minutes. Homogenized protein samples were separated by electrophoresis on a polyacrylamide gel and proteins were transferred to PVDF (Millipore, MA, USA) and NC (GE healthcare, IL, USA) membranes. The following primary antibodies were used: mouse anti-β-actin (1:5000, A5316, Sigma-Aldrich, MA, USA), rabbit anti-αSMA (1:1000, ab5694, Abcam, Cambridge, UK), rabbit anti-p300 (1:1000, ab61217, Abcam, Cambridge, UK), rabbit anti-COL1A (1:1000, sc59772, Santa Cruz Bio-technology, TX, USA), mouse anti-CTGF (1:1000, sc65970, Santa Cruz Biotechnology, TX, USA), rabbit anti-fibronectin (1:1000, ab2413, Abcam, Cambridge, UK), rabbit anti-periostin (1:1000, ab14041,

Abcam, Cam-bridge, UK) and rabbit anti-tenascin C (1:1000, ab108930, Abcam, Cam-bridge, UK). The secondary antibodies used were: anti-mouse secondary antibody (1:5000, 31430, Thermo Fisher Scientific, MA, USA) and an-ti-rabbit secondary antibody (1:5000, 31460, Thermo Fisher Scientific, MA, USA). Chemiluminescence signals were visualized on the Fusion SOLO S device (Vilber, Marne-la-Vallée, France) and quantified using ImageJ software.

8. LX2 cell culture

LX2 cells were gifted by Dr. Jae-woo Kim (Yonsei University College of Medicine, Korea). Cells were maintained in DMEM (Corning, NY, USA) supplemented with 10% FBS and 1% antibiotic-antimycotic solution. Cells were incubated at 37 ° C and 5% CO2. For experimental procedures, cells were first starved in serum-free media for 2 hours and then stimulated with 20 ng/ml TGF-β1 (Prospec, Rehovot, Israel). Cells were treated with either 3 uM C646 (Selleckchem LLC, TX, USA) or 3 uM A6 and then collected 24 hours after treatment.

9. Liver sample preparation for histopathological staining

Mouse livers were isolated under anesthesia and fixed in ice-cold 10% paraformaldehyde for 24 hours. After fixation, liver tissue was embedded in paraffin and sectioned at a thickness of 4 um. Liver sections were de-paraffinized with xylene and rehydrated through 100%, 90%, and 80% ethyl alcohol series prior to staining as listed below.

10. Masson's trichrome staining (MTS)

Deparaffinized liver sections were stained using Wiegert's Iron Hematoxylin Set (Sigma-Aldrich, MA, USA) and MTS Kit (Sigma-Aldrich, MA, USA) according to the manufacturer's instructions.

11. Sirius red staining

Deparaffinized liver sections were stained using the Picro-Sirius Red Stain Kit (Abcam, Cambridge, UK). Liver sections were submerged in Picro-Sirius Red solution for 2 hours at room temperature, washed twice with 5% acidified water, and then washed twice with absolute alcohol. After staining, sections were mounted using a hydrophilic mounting solution.

12. Immunohistochemical staining (IHC) and immunofluorescences staining (IF)

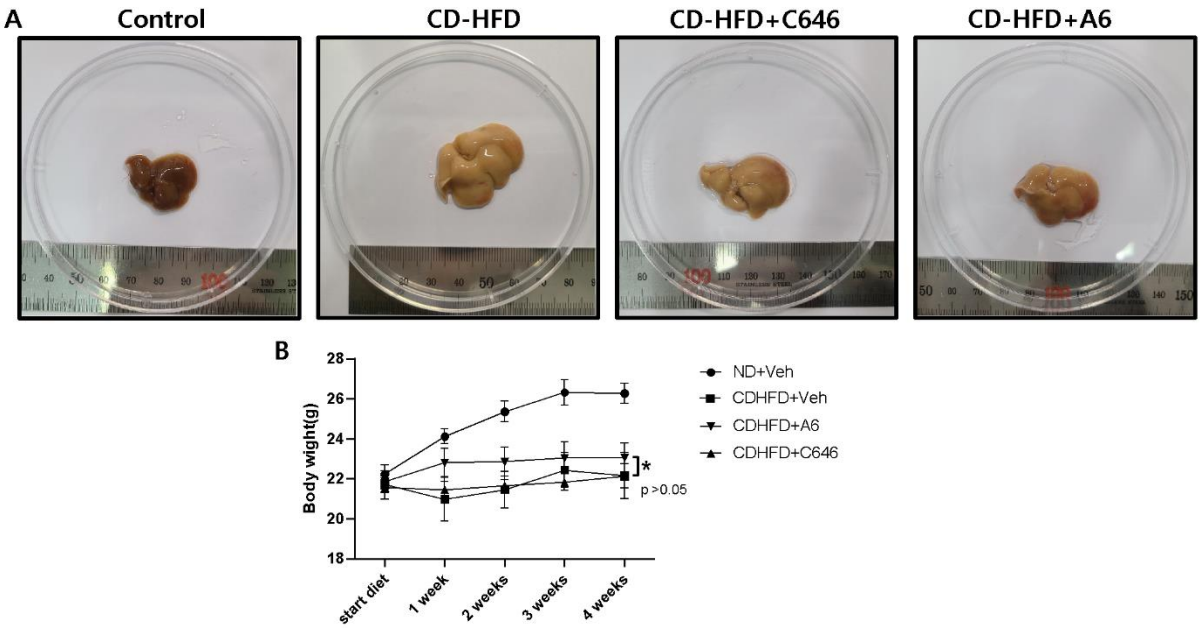
Deparaffinized mouse liver sections were treated for anti-gen-retrieval (Antigen Unmasking solution, Citric acid base, Vector Laboratories, CA, USA) in the microwave at 4 minutes followed by a peroxidase blocking step (Dako REAL Peroxidase-blocking Soltion, Dako, Glostrup, Denmark). The tissue samples were incubated with a primary antibody against α-smooth muscle actin (1:500, αSMA, Abcam, Cam-bridge, UK) or p300 (1:200, p300, Cell Signaling, MA, USA). For IHC, primary antibody staining was followed by HRP-conjugated anti-rabbit secondary antibody (Envision+ system-HRP Labelled Polymer anti-rabbit, Dako, Glostrup, Denmark) and visualized by 3'- diaminobenzidine substrate (Vector Laboratories, CA, USA). For IF, αSMA and p300 primary antibody staining were followed by Dylight 488 anti-mouse antibody (1:500, Vector Laboratories, CA, USA) and Dylight 549 anti-rabbit anti-body (1:500, Vector Laboratories, CA, USA).

13. Chromatin immunoprecipitation-qPCR (ChIP-qPCR)

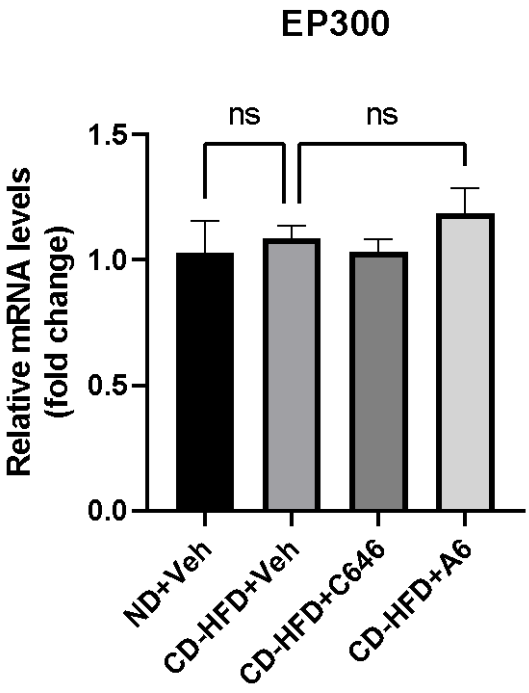
Chromatin immunoprecipitation was performed using a Pierce Agarose ChIP kit (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions. The antibody used for precipitation was anti-p300 (Santa Cruz Biotechnology, TX, USA) and anti-IgG (Thermo Fisher Scientific, MA, USA) for normalization. RT-qPCR analyses were per-formed using FastStart Universal SYBR Green Master (ROX) master mix (Roche, Basel, Switzerland) and the ABI Prism 7700 sequence detection system (Applied Biosystems, CA, USA). The primer sets used for amplification are listed in Supplementary Table 2.

14. Statistical analysis

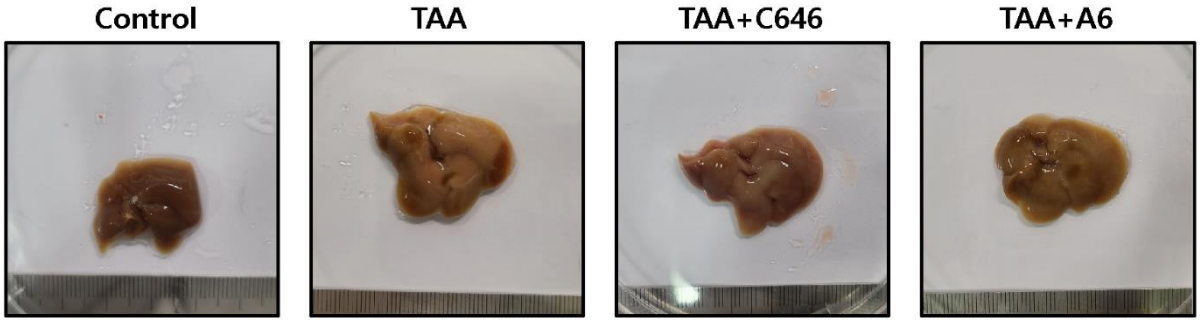
Statistical analyses were performed by one-way ANOVA (comparisons of more than two groups, using Mann Whitney or Kruskal Wallis analyses) and T-test analyses (comparisons of two groups). Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software Inc, La Jolla, CA, USA). A p-value of < 0.05 was considered a statistically significant difference.



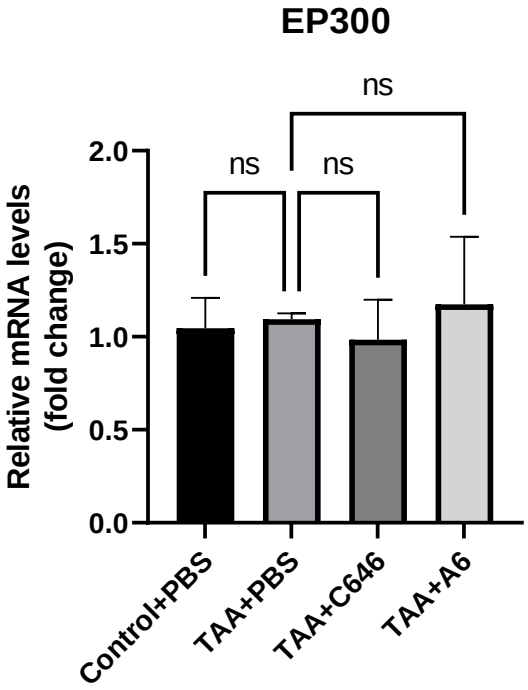
Supplementary Figure 1. Liver morphology and change of body weight in CD-HFD induced liver fibrosis model. (A) Representative images of livers from mice with or without p300 inhibitor at 4 weeks after starting CD-HFD diet. (B) Change of body weight in CD-HFD induced liver fibrosis model mouse (n=5). Body weight was measured at 1, 2, 3, and 4 weeks after starting the CD-HFD diet. Data are represented as mean ± SEM, *p 〈 0.05, compared with the vehicle-injected group by ordinary one-way ANOVA test.



Supplementary Figure 2. mRNA expression levels of p300 in CD-HFD-induced liver fibrosis treated with A6. RT-qPCR analysis of expression of fibrosis marker gene *EP300* in the liver of mice with or without p300 inhibitors at 4 weeks after CD-HFD (n = 5). Data are represented as mean ± SEM, ns = not significant by ordinary one-way ANOVA test.



Supplementary Figure 3. Liver morphology in TAA-induced liver fibrosis model. Representative images of livers from mice with TAA-induced liver fibrosis with or without p300 inhibitor at 2 weeks after TAA injection.



Supplementary Figure 4. mRNA expression levels of p300 in TAA-induced liver fibrosis treated with A6. RT-qPCR analysis of expression of *EP300* in the liver of mice with or without p300 inhibitors at 2 weeks after starting TAA administration (n = 5). Data are represented as mean ± SEM, ns = not significant by ordinary one-way ANOVA test.

Supplementary Table 1. Primer sequences for RT-qPCR

Gene name (Forward: F; Reverse: R)	Sequence 5'-3'
human_COL1A1_F	TCCTGCTGGTGAGAAAGGAT
human_COL1A1_R	TCCAGCAATACCCTGAGGTC
human_COL3A1_F	TGGTCTGCAAGGAATGCCTGGA
human_COL3A1_R	TCTTTCCCTGGGACACCATCAG
human_CTGF_F	CAAGGGCCTCTTCTGTGACT
human_CTGF_R	ACGTGCACTGGTACTTGCAG
human_TNC_F	CAGAAGCCGAACCGAAGTT
human_TNC_R	TTCATCAGCTGTCCAGGACAGA
human_ACTA2(aSMA)	CTGGCATCGTGCTGGACTCT
human_ACTA2(aSMA)	GATCTCGGCCAGCCAGATC
human_EP300_F	TTGTGAAGAGCCCCATGGAT
human_EP300_R	GCTTTGCATCACTGGGTCAA
human_FN_F	AAGACCAGCAGAGGCATAAGG
human_FN_R	TGTAGGGGTCAAAGCACGAG

Supplementary Table 2. : Primer sequences for ChIP-qPCR assay

Gene name (Forward: F; Reverse: R)	Sequence 5'-3'
human_COL1A1_F	GAGATGGCATCCCTGGAC
human_COL1A1_R	CCCATTGGACCTGAACCG
human_COL1A2_F	CTGGACTTCCTGGCTTCAA
human_COL1A2_R	AGTTCACCCTTGGGACCAG