Master's Thesis

# Anti-obesity effects of D6 via activation of PPARs

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# PPARs 활성화를 통한 D6의 항비만 효과에 관한 연구

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by

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# Abstract

Obesity can cause type 2 diabetes, non-alcoholic fatty liver disease (NAFLD), hypertension and several metabolic diseases. As obesity is a fundamental cause of metabolic diseases, obesity is the primary factor for treatment in the prevention of metabolic diseases. The key treatment for obesity is the suppression of appetite by the activation of the 5-HT receptor. However, there is a risk affecting the Central Nervous System (CNS), which can lead to several mental side-effects. PPARs are attracting attention as therapeutic targets of metabolic diseases because of their ability to control glucose and lipid metabolism in liver, adipose tissue, muscle and so on. In particular, agonists of PPARα are recently known to have great potential to alleviate NAFLD. Our data suggest that chemical compound D6 is a novel PPAR pan agonist with preferential activation on PPARα. D6 decreases the lipid droplets increased by roglitazone in adipocytes 3T3-L1. In addition, high fat diet (HFD) fed mice treated with D6 (HFD-D6) showed weight loss and improvement of glucose control. In HFD-D6 mice, liver lipid droplets were slightly reduced and NAFLD was alleviated. Our data indicate that D6 has anti-obesity, anti-diabetic and anti-NAFLD effects. In this regard, D6 may have huge potential to be developed as a drug for obesity, diabetes, NAFLD and other metabolic diseases.

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## ABBREVIATIONS

| | |
|---|---|
| WHO | World health organization |
| T2DM | Type 2 diabetes mellitus |
| CVD | Cardiovascular disease |
| NAFLD | Non-alcoholic fatty liver disease |
| 5-HT | 5-Hydroxytryptamine (serotonin) |
| CNS | Central nervous system |
| PPAR | Peroxisome proliferator-activated receptor |
| RXR | Retinoid X receptor |
| PPRE | PPAR response elements |
| hPPAR-LBD | His-tagged recombinant human PPAR-Ligand Binding Domain |
| NASH | Non-alcoholic steatohepatitis |
| HFD | High fat diet |
| GTT | Glucose tolerance test |
| ITT | Insulin tolerance test |
| AUC | Area under curve |
| H&E | Hematoxylin and eosin |
| ALP | Alkaline phosphatase |
| LDLC | Low-density lipoprotein cholesterol |
| HDLC | high-density lipoprotein cholesterol |
| TG | Triglyceride |
| CHOL | Cholesterol |
| HSL | Hormone sensitive lipase |
| ATGL | Adipose triglyceride lipase |
| HPGA | Hypothalamic pituitary gonadal axis |

# I . INTRODUCTION

It has become obvious that the prevalence of obesity has been rapidly increasing in not only South Korea but also worldwide.[1,2] Therefore, the World Health Organization (WHO) has defined obesity as a disease, not just a phenomenon of individuals since 1996.[3] There are a number of reasons why obesity has gained attention worldwide. People want to lose weight not only for beauty but also for health. Since obesity is accompanied by several metabolic disorders, obesity imposes an economic burden on both individuals and the government.[4,5] Obesity-induced diseases include Type 2 Diabetes Mellitus (T2DM), high blood pressure, Cardiovascular Disease (CVD), Non-alcoholic Fatty Liver Disease (NAFLD), cancer and so on.[2] These complications due to obesity are the leading cause of death.[6] Obesity is the fundamental cause of the complications. Therefore, obesity must be treated to cure obesity-induced diseases. Drugs currently used to treat obesity mainly act on the 5-hydroxytryptamine (5-HT, serotonin) receptor. Thus, they are involved in the Central Nervous System (CNS) and suppress appetite.[7] Because these appetite suppressants act directly on the CNS, there are risks of side-effects such as nausea, headache, insomnia, mood changes, etc.[7,8] Therefore, it is important to develop new drugs that minimize these mental side-effects.

We selected the Peroxisome Proliferator-Activated Receptor (PPAR) as a target for the development of a new anti-obesity agent. The background for selecting PPAR results from the intracellular function of PPAR. PPAR regulates glucose and lipid metabolism in the liver, adipose tissue, muscle and so on.[9,10] There are three subtypes of PPAR: PPARα, PPARδ, and PPARγ. PPARα is mainly involved in regulating lipid metabolism and PPARγ is primarily influenced in controlling glucose metabolism.[11] PPARs are activated when certain ligands bind and active PPARs form heterodimers with Retinoid X Receptor (RXR).[12] PPAR-RXR heterodimer

can bind to the specific sequence PPAR Response Elements (PPRE) of DNA.[12] In combination with PPRE, PPAR acts as a transcription factor that regulates the transcriptional expression level of the target gene.[10,11] The target genes of PPARs have some differences by subtype, but they are generally related to energy metabolism. [11,13] Therefore, there is an advantage that it can control the overall energy metabolism by targeting PPARs. Besides, PPARs inhibit inflammation via the NF-κB pathway.[14] PPAR agonists do not act as a neurotransmitter, so there is low possibility of mental adverse side-effects such as insomnia, neurological and psychological abnormalities. Thus, PPAR agonists are attractive due to their mild side-effects and alleviation of obesity, T2DM, NAFLD and other obesity-induced diseases. Recently clinical studies of PPAR pan agonist (chiglitazar and lanifibranor) have begun as treatments for T2DM.[13] In this study, we present D6 as a new PPAR pan agonist and suggest the possibility of using D6 as a therapeutic agent for obesity, T2DM, NAFLD, and other metabolic diseases. We have performed several animal experiments to confirm the efficacy of D6.

# Ⅱ. MATERIALS AND METHODS

### Chemicals and Reagents

All reagents used for our study were analytical grade. D6 was synthesized through DAEJUNG Chemicals & Metals (Gyeonggi-do, Korea). WY14643, GW501516, rosiglitazone, and etoposide were purchased from Sigma-Aldrich (St. Louis, MO, USA). These compounds were resolved in dimethyl sulfoxide (DMSO) Bioshop Canada (Burlington, Ontario, Canada) and diluted with culture medium before the experiment. Cyanogen bromide (CNBr)–activated Sepharose 4B was purchased from Sigma-Aldrich (St. Louis, MO, USA). His-tagged recombinant human PPAR-Ligand Binding Domain (hPPAR-LBD) were expressed in E.coli (DE3, Rosetta) and purified. His antibody was purchased from Cell Signaling Technology (Danvers, Massachusetts, USA). 3-Isobutyl-1-methylxanthine (IBMX), dexamethasone, insulin from bovine pancreas, Oil Red O was purchased from Sigma-Aldrich (St. Louis, MO, USA). PRO-PREP™ Protein Extraction Solution was purchased from iNtRON Biotechnology (iNtRON Biotech., Korea). Actin, Hormone Sensitive Lipase (HSL), and Adipose Triglyceride Lipase (ATGL) antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Comet Assay Kits, 96-Well was purchased from Cell Biolabs (San Diego, CA, USA)

### Plasmids

All plasmids used for transformation to E.coli (DE3, Rosetta) and transfection to human embryonic kidney 293 (HEK293) cells were cloned in our laboratory. Three types of pcDNA5-GAL4 containing PPARα-LBD, PPARδ-LBD or PPARγ-LBD, pGALRE-luc were received from the Faculty of Pharmaceutical Sciences, Toho University and used for luciferase assay[15,16]. pProEX-PPAR and pET-28a were used

for His-tagged recombinant hPPAR-LBD protein purification.

**Cell culture**

HEK293 cell line was grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin (P/S). Mouse preadipocyte 3T3-L1 cell line was maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% Calf Serum (CS) and 1% P/S. For differentiation of 3T3-L1, DMEM with 10% FBS and 1% P/S were used. These cell lines were grown in 37℃ incubator humidified atmosphere of 5% $CO_2$.

**Cell viability assay (MTT assay)**

To measure HEK293 and 3T3-L1 cell viability after treatment of drugs for 24 hours, we used 3-(4, 5-dimethyltiazol-2-ly)-2, 5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's instruction.

**Single-cell gel electrophoresis Comet assay**

To measure DNA damage of HEK293 and 3T3-L1 cells after treatment of drugs for 4 hours, we used Comet Assay Kits, 96-Well (Cell Biolabs, San Diego, CA, USA) according to the manufacturer's protocol. The images of Comet assay were calculated by CometScore 2.0.

**Transfection**

HEK293 was transfected by microporation using MP-100 microporator (Invitrogen,

Carlsbad, CA, USA) according to the manufacturer's protocol.

**Luciferase assay**

After the treatment of drugs for 24 hours to transfected HEK293 cells, the cells were harvested and resuspended in luciferase lysis buffer (Promega, Madison, Wisconsin, USA), followed by incubation on ice for 10 minutes. Cell debris was removed by centrifugation at 15000rpm, 4℃ for 10 minutes. Then, supernatants were used for the luciferase assay. Firefly Luciferase (Fluc) and Renilla Luciferase (Rluc) activities were determined using the Dual Luciferase Reporter Assay System (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions. The ratio of Fluc to Rluc activity was defined as the activity of Ligand Binding Domain (LBD) of PPAR.

**CNBr-bead conjugation assay**

100mg of CNBr-activated Sepharose 4B was added to an e-tube (being the equivalent of 20mg per CNBr-bead conjugation reaction, so that 5 reactions could be carried out per e-tube). After, the beads were activated by rotating the e-tubes with 1ml of 1mM HCl for over 10 minutes at room temperature. Over 10 minutes, change the solution to a new one and repeat it. The supernatants were removed by centrifugation at 5000rmp for 1 minute. Then samples were washed with Coupling buffer and repeated three times. The samples were separated into 20mg of the bead in other e-tubes. Each e-tube was added with drugs and rotated over-night at 4℃. The concentration was 20 μM. The next day, samples were washed with Coupling buffer three times. After washing, the samples were blocked with 1ml of Blocking buffer room temperature for 2 hours. After blocking, the samples were washed with Washing buffer 1 three times, repeated with Washing buffer 2

and Pull-down buffer in order. Then, the supernatants were removed by centrifugation at 5000rmp for 1 minute. The pellets were added 0.8ml of Pull-down buffer and purified protein and rotated over-night at 4℃. Finally, the samples were washed with Pull-down buffer three times. The supernatants were removed completely. The pellet would be detected by western blot. This experiment was accord to GE Healthcare's protocol.

**In silico docking analysis**

Crystal structures of the LBD of human PPAR family (PPARα: 4BCR, PPARδ: 5U46, PPARγ: 5YCP) were prepared from RCSB Protein Data Bank. The energy of D6 or ligands of PPARs (WY14643, GW501516 and Rosiglitazone) were minimized using open babel in PyRx software for further docking analysis.

**Differentiation of 3T3-L1**

The differentiating experiment was accorded to the 3T3-L1 differentiation protocol by Robin Erickson. First, cells were seeded in 12 well plates at a density of $5 \times 10^4$ cells/well. The cells were grown in DMEM with 10% CS and 1% P/S until the confluency was full. Two days after 100% confluency, the cells were treated with MDI (0.5mM IBMX, 1μM dexamethasone, 1μg/ml insulin) media, which used DMEM with 10% FBS and 1% P/S. Two days after MDI, the media was changed to Insulin (1μg/ml insulin). Two days later the media was changed to DMEM with 10% FBS and 1% P/S, and treated with the drugs. The media was changed to a new DMEM with 10% FBS, 1% P/S and the drug every two days three times. Finally, the media was changed to DMEM only with the drug for serum starvation. After two days, the differentiation of 3T3-L1 was finished.

**Oil Red O stain**

After differentiation of 3T3-L1 in 12 well plates, the cells were fixed by 4% paraformaldehyde, then stained the Oil Red O solution. Pictures of the sample with finished staining were taken using a microscope (Nikon Eclipse Ti-s). The Oil Red O dye of the sample was dissolved in isopropanol and detected with a spectrometer at 492nm. This experiment was referred to the Lonza's document # WEB-PR-PT-2501OIL-3 and BioVision's protocol. The OCT-embedding liver samples were stained by Roy Ellis's protocol.

**Western blot analysis.**

After the treatment of drugs to differentiated 3T3-L1 cells, the cells were harvested, then resuspended with PRO-PREP™ Protein Extraction Solution (iNtRON Biotechnology, Korea). The samples were incubated in ice for 10 minutes, vortexed 2 seconds, and incubated in ice for 10 minutes again. After centrifugation (15000rpm, 20 minutes, 4℃), protein concentration was determined by Bradford Assay with 1mg/ml BSA. The same amount of proteins were separated on SDS-polyacrylamide gel and the electrically blotted to polyvinylidene difluoride membrane (PVDF) (Millipore, Billerica, MA, USA). The blots were blocked by blocking solution (5% BSA in TTBS) at room temperature for 30 minutes and then incubated at 4℃ overnight with specific primary antibodies, anti-HSL, anti-ATGL, and anti-Actin. Then the blots were incubated with secondary antibodies, at room temperature for 2 hours. The signal was detected by LAS4000.

**Animal**

Animals care and use was approved by the Animal Care and Use Committees of Pohang University of Science and Technology. C57BL/6J mouse strain was

purchased from Japan SLC, Inc. Fifty 4-weeks old male C57/BL/6J mice were adapted for 2 weeks. The mice were randomly treated with vehicle or 5 mg/kg/day of D6 or WY14643 by oral gavage for 13 weeks and induced obese by 60% high-fat diet (#D12492, Research diets, NJ, USA). Body weight and food intake measurements were taken weekly from 6 weeks of age. After 16 weeks of age, the volume of adipose tissue and muscle were measured by the Trimodality imaging system.

**Intraperitoneal Glucose Tolerance Test (IP-GTT)/ Insulin Tolerance Test (IP-ITT)**

After 16 weeks of age, GTT were performed after 16 hours fasting with intraperitoneal administration of glucose (2g/kg, body weight). Blood glucose levels were measured 0, 15, 30, 45, 60, 90, and 120 minutes after injection via tail vein blood. After 17 weeks of age, ITT were performed after 3 hours fasting with intraperitoneal administration of insulin (0.75U/kg, body weight). Blood glucose levels were measured 0, 15, 30, 45, 60 and 90 minutes after injection via tail vein blood.

**Histology**

Mouse tissues fixed by Formalin solution, neutral buffered, 10% (Sigma-Aldrich, St. Louis, MO, USA). Paraffin-embedding tissues were sectioned and subject to Hematoxylin and Eosin (H&E) staining in the Division of Animal Facility & Histopathology Lab (POSTECH, Korea). All bright-field images were taken by using the Nikon Eclipse Ti-s microscope. Paraffin-embedding tissues were stained by NovaUltra Sirius Red stain kit (IHC world, Woodstock, USA). Fibrosis and vesicular fat in the liver tissues were confirmed using Sirius Red and Oil Red O staining. The NAFLD activity score was evaluated semi-quantitatively according to NASH Clinical

Research Network Scoring System.[17]

**Serum analysis.**

Mouse blood was gained through heart puncture. The blood samples were incubated at room temperature for over 30 minutes. Then they were centrifuged at 3000rpm, 4℃ for 30 minutes. The supernatants were collected for analysis. Serum was subject to analysis in Pohang Technopark (Pohang, Korea).

**Statistical analysis**

All data are expressed as the mean ± standard error of the mean (SEM). Statistical significance was measured by one-way ANOVA using GraphPad Prism 6.0

# Ⅲ. RESULTS

## 3.1 D6 has low cytotoxicity and DNA damage at the mainly used concentration (25μM) in HEK293 and 3T3-L1.

Low toxicity is essential for synthetic chemicals to be used as drugs. Two toxicity tests in the cell were performed to confirm that the synthetic chemical D6 was available as a therapeutic agent. One was MTT, which measured cell viability, and the other was Comet assay for quantification of DNA damage. MTT assay showed that cell viability of HEK293 and 3T3-L1 decreased at high concentrations, being 100μM for all drugs that we used in this study (Fig. S1A, B). However, there was no difference in cell viability between vehicle-only and D6 concentrations under the 25μM, being the low concentration drugs that were used in this study. Also, it was shown that the Comet assay conducted at 50μM did not significantly affect DNA damage (Fig. S1C-J). The results of the Comet assay showed that there was no difference in the degree of DNA damage of D6 compared to DMSO (vehicle-only) (Fig. S1D-F, H-J).

## 3.2 D6 is a pan-agonist of PPARs

To find an agonist of PPAR, we tested a chemical library via Gal4 trans-activating luciferase assay. When D6 was treated, the relative luciferase activity increased dose-dependently and positively (Fig. 1A-C). In PPARα, the rise of D6 luciferase activity was higher than that of positive control, WY14643 (Fig. 1A). In PPARδ and PPARγ, the luciferase activity of D6 was not greater than the positive controls, GW501516 and Rosiglitazone, respectively. Both were slightly but significantly increased (Fig. 1B, C). CNBr-bead conjugation experiments were performed to verify that D6 bound directly to PPAR (Fig. 1D-F). D6 and each PPAR agonist (being

WY14643, GW501516 and Rosiglitazone) were coupled to CNBr, reacted with His-tagged recombinant human PPAR-Ligand Binding Domain (hPPAR-LBD) and confirmed their binding to PPAR by the Western bolt. Free drugs, which were not conjugated with CNBr, acted as competitors of the CNBr-coupled drugs. When the CNBr-coupled drugs that had reacted with the hPPAR-LBD were mixed with the free drugs, the bands disappeared. It was also found that D6 bound to all three subtypes of PPAR as strong as the positive controls. In-silico analysis was used to predict the binding site of the D6 in ligand-binding domain (LBD) of PPARs and binding energy of D6 with LBD of PPARs (Table 1-3). According to Tables 1-3, the binding energy of D6 was similar to representative agonists of PPARs.

### 3.3 D6 decreases the lipid content of rosiglitazone-treated mature 3T3-L1.

It is well-known that PPARs are primarily involved in regulating energy metabolism.[11,13] We planned a 3T3-L1 differentiation experiment for confirming the effect of D6 in 3T3-L1. 3T3-L1 was treated with D6 during differentiation to determine whether metabolism changes in 3T3-L1 due to the effect of D6, which acts as a PPAR pan-agonist. Lipid droplets in D6 treated differentiated 3T3-L1 were similar to DMSO (Fig. 2A). In order to quantitatively measure the amount of lipid, Oil Red O dye was dissolved with isopropanol and was measured at OD 492nm. This suggested that the amount of lipid in D6 was lower than that of Rosiglitazone (Fig. 2B). Treatment with rosiglitazone alone increased the size of large lipid droplets while reducing the size of small lipid droplets (Fig. 2C). When rosiglitazone and D6 were treated together, the effect of rosiglitazone was lost (Fig. 2C). In other words, D6 effectively reduced the size of lipid droplets in 3T3-L1 adipocytes.

### 3.4 D6 inhibits increased body weight and relieves energy metabolism in mice.

To determine whether the lipid-lowering effect of D6 seen *in vitro* is retained *in vivo*, high fat diet (HFD) fed mice were treated with D6 (We called this group HFD-D6). Several animal experiments were performed according to the schedule as shown in Figure 3A and outlined under 'Animal' in the Materials and Methods section above. Body weight gain in the HFD-D6 remarkably decreased compared to the control HFD-V (Fig 3B, C). There was no significant difference in the amount of food intake (Fig. 3D). In addition, when the volume of adipose tissue was measured by micro-CT, it was clearly reduced (Fig. 3E, F). There was no muscle difference between these groups of mice (Fig. 3G). We could assume that D6 had no significant effect on muscles. The weight of epididymal white adipose tissue (eWAT) tended to decrease in HFD-D6 (Fig. 3H). The eWAT weight (mean ± SEM) of NC-V, NC-D6, HFD-V, HFD-D6 and HFD-WY were 0.81 ± 0.16 g, 0.62 ± 0.07 g, 3.55 ± 0.32 g, 2.91 + 0.4 g, and 2.00 ± 0.29 g, respectively. The eWAT weight was slightly reduced in HFD-D6. There was no difference between all mice groups in the weight of the spleen, kidney, and heart, but in the liver, HFD-D6 had decreased weight compared to HFD-V. Therefore, it confirmed that D6 alleviated the symptoms associated with the liver. Furthermore, Glucose Tolerance Test (GTT) and Insulin Tolerance Test (ITT), which are related to metabolism, showed that D6 had the ability to relieve the symptoms of diabetes (Fig. 3I-L). Serum glucose levels also decreased (Fig. 3M). Interestingly, no lipids showed any significant change except for LDLC, which is considered as bad cholesterol. The level of LDLC reduced significantly (Fig. S2A-C, Fig. 3O). This suggested that the overall symptoms of the metabolic disease had been alleviated in HFD-D6. Hematoxylin and Eosin (H&E) images of adipose tissues showed a reduction in the size of adipocytes (Fig. 4A). This was equally observed in inguinal WAT (iWAT) and eWAT, and brown adipose tissue (BAT). The H&E images were analyzed by MATLAB and the size of adipocyte

was significantly reduced in eWAT not iWAT. (Fig. 4B, C).

### 3.5 D6 alleviates HFD-induced NAFLD.

The number of NAFLD and Non-Alcoholic Steatohepatitis (NASH) patients has grown rapidly since the early 2000s.[18] For example, annual rates of incident NAFLD diagnoses in the U.S. increased 12-fold between 2000 and 2017.[19] But there is no FDA-approved NASH treatment. This is why large pharmaceutical companies have been interested in NAFLD and tried to develop NASH drugs. Since liver weight was reduced in HFD-D6 (Fig 3H), we planned liver histologic experiments to investigate whether D6 could be a potential treatment for NAFLD. In addition, it was necessary to check the toxicity of liver tissue. Fatty liver and fibrosis were confirmed by histologic stainings such as H&E, Oil Red O, and Sirius Red (FIG. 5A). By the images of H&E, the NAFLD activity score was assessed according to the NASH Clinical Research Network Scoring System.[17] In the HFD-D6 group, the NAFLD activity score was lower than the HFD-WY group, which was a positive control (FIG. 5B). In Sirius Red, the effect of D6 could not be determined because complete fibrosis had not occurred by 10 weeks of HFD. Histological imaging of lipid droplets in the liver by Oil Red O showed that D6 caused a minor decrease in the amount of lipid droplets in the liver by percentage area, although this was not significant (Figure 5C).

### 3.6 There is little difference between other organs of HFD-V and HFD-D6.

The Alkaline Phosphatase (ALP), which is a marker of liver damage, did not differ in NC-V and HFD-D6. D6 did not cause severe hepatotoxicity, unlike other small compounds (Fig. S3A) and there is no significant difference in the levels of blood urea and creatinine in serum, suggesting there is no significant toxicity in the kidney (Fig. S3B, C). In addition, it was revealed that there is no significant

difference in heart and kidney (Fig. S3D). Therefore, it is proposed that D6 has high potential as a therapeutic agent for obesity and obesity-induced NAFLD because D6 acted as a pan agonist of PPAR and had low side-effects.

# IV. DISCUSSION

Obesity is a serious concern worldwide as it is accompanied by a wide range of diseases, for example, T2DM, heart diseases, NAFLD, etc.[1,2] PPAR family was considered as a target gene for the treatment of obesity because PPAR is one of the transcription factors that regulate overall lipid and glucose metabolism.[9] There are some advantages in approaching PPARs as a therapeutic strategy for obesity.[20] Firstly, PPAR agonists possess no anabolic activity and do not bind to the androgen receptor. Secondly, PPAR agonists do not suppress Hypothalamic–Pituitary–Gonadal Axis (HPGA) or affect testosterone production. Thirdly, PPAR agonists can control both lipid and glucose metabolism and inflammation. Finally, PPAR agonists do not affect sleep or cortisol levels. Thus, researchers have tried to find a candidate molecule for obesity among several PPAR agonists.[21] Only fenofibrate and gemfibrozil among PPARα agonists have been approved by the FDA for treating dyslipidemia.[13] WY14643 is a PPARα agonist used only in research.[22] GW501516, which is a PPARδ agonist, have anti-obesity effects. However, it was halted in 2007 due to carcinogenesis and resumed in 2011.[21] There is no FDA-approved drug among PPARδ selective agonist yet. Agonists of PPARγ (thiazolidinediones, rosiglitazone and pioglitazone) are FDA-approved for the treatment of T2DM.[13] The ligands of PPARα and PPARδ are well-known for dyslipidemia and weight loss, respectively.[23] On the other hand, these PPARγ ligands are mostly effective for T2MD, but there are some problems, especially weight gain.[24] There have been several attempts to activate all the subtypes of PPAR simultaneously for their complementary functions, using PPAR dual, pan agonists or a combination of two or more drugs in order to treat metabolic diseases efficiently.[13] PPAR pan agonists have greater control over metabolism than the selective agonists of the three PPAR subtypes. The treatment with a pan

agonist can also be more effective since there are various causes of metabolic disorders. Chiglitazar and lanifibranor are currently conducting Phase III clinical studies of PPAR pan agonist as treatments for T2DM.[13]

In this study, we found that D6 is a potential therapeutic agent for obesity since it has shown to be a new pan agonist for PPAR. It is also suggested that D6 be used as a potential new treatment for obesity-induced metabolic disease, especially NAFLD because D6 binds to and activates all subtypes of PPARs, predominantly activating PPARα (Fig. 1, Table 1-3). There have been no reports of synthetic compound D6. The results of our cell and mouse experiments reveal that D6 inhibits obesity (Fig. 2-5). Accordingly, pre-clinical animal studies demonstrate D6 as a potential anti-obesity treatment.

In order to find out the mechanism by which D6 inhibits obesity, we have carried out a preliminary *in vitro* experiment (Fig. S4). The addition of D6 in 3T3-L1 caused an increase in expression of the lipase ATGL, therefore increasing lipolysis. Furthermore, although there was also a slight increase in the expression of HSL, this was not a significant increase. This has provided an insight into the mechanism of D6 *in vitro*. However, the *in vivo* and intracellular pathway after PPAR activation by D6 treatment is not well-known. Investigation on the molecular mechanisms of D6 is needed to further explore the effects of D6 on genes involved in lipid and glucose metabolism, like Fatty Acid β-Oxidation (FAO), lipolysis, lipogenesis, lipid and glucose transport, adipogenesis, and so on. In addition, more toxicological tests must be carried out prior to confirming the use of D6 as a therapeutic drug although our animal study revealed that there is no significant effect of D6 on liver, heart and kidney toxicity (Fig. S1, S3).

In summary, we propose D6 as a potential alternative treatment for anti-obesity and NAFLD therapy, although further investigation into the mechanism and safety of D6 *in vivo* and pre-clinical trials, amongst others, are necessary.

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# VI. FIGURES

## Figure 1.

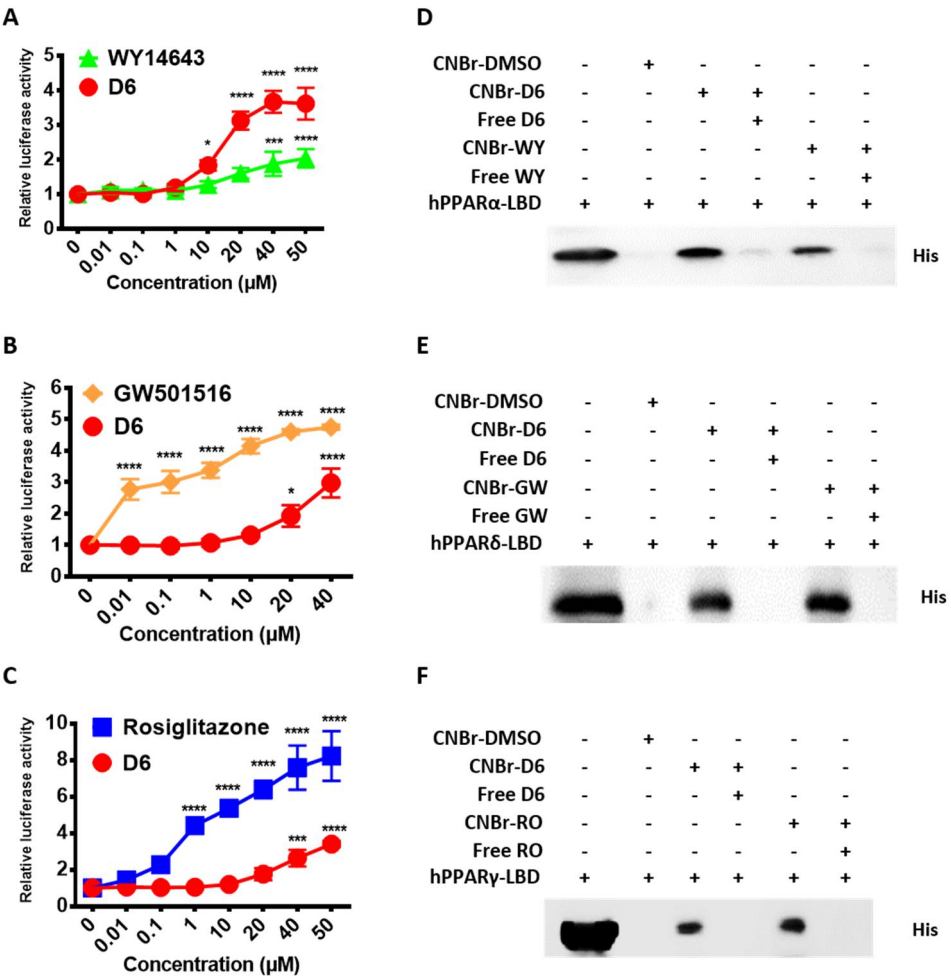


**Figure 1. D6 activates PPARs and binds to LBD of all PPARs.**

(A-C) D6 activates the transcriptional activity of PPARs. HEK293 cells were transfected with the PPAR Gal4-luciferase reporter system, treated for 24 hours, harvested and analyzed by Dual-Luciferase assay. Fluc activity was normalized to Rluc activity. (A), (B) and (C) show PPARα, PPARδ and PPARγ respectively. WY14643, GW501516 and rosiglitazone are representative PPARα, PPARδ and PPARγ agonist respectively. They are used by positive control. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001 (versus 0, DMSO), All subtypes of PPAR luciferase assays were repeated at least three times in independent experiments. Data represent the mean ± standard error of means (SEM) and statistical significance was tested by one-way ANOVA followed by Dunnett's multiple comparisons test. (D-F) Recombinant human PPARs' LBD protein directly bound to the D6-conjugated CNBr-bead. DMSO is a vehicle, negative control. 'Free drugs' mean not conjugated CNBr-bead WY, GW and RO mean WY14643, GW501516 and rosiglitazone respectively. (D), (E) and (F) show PPARα, PPARδ and PPARγ respectively. N=3 independent experiments.

**Figure 2.**

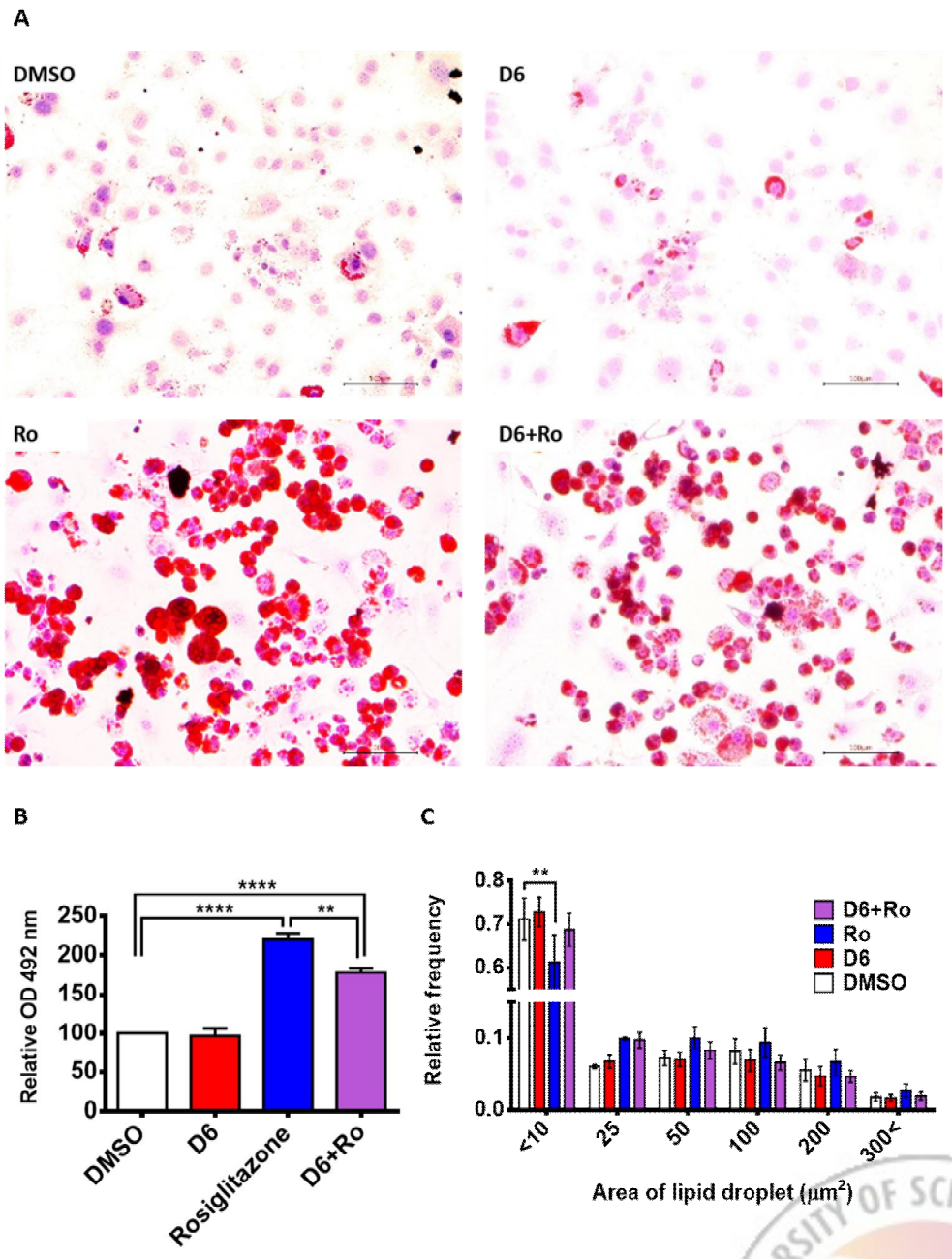
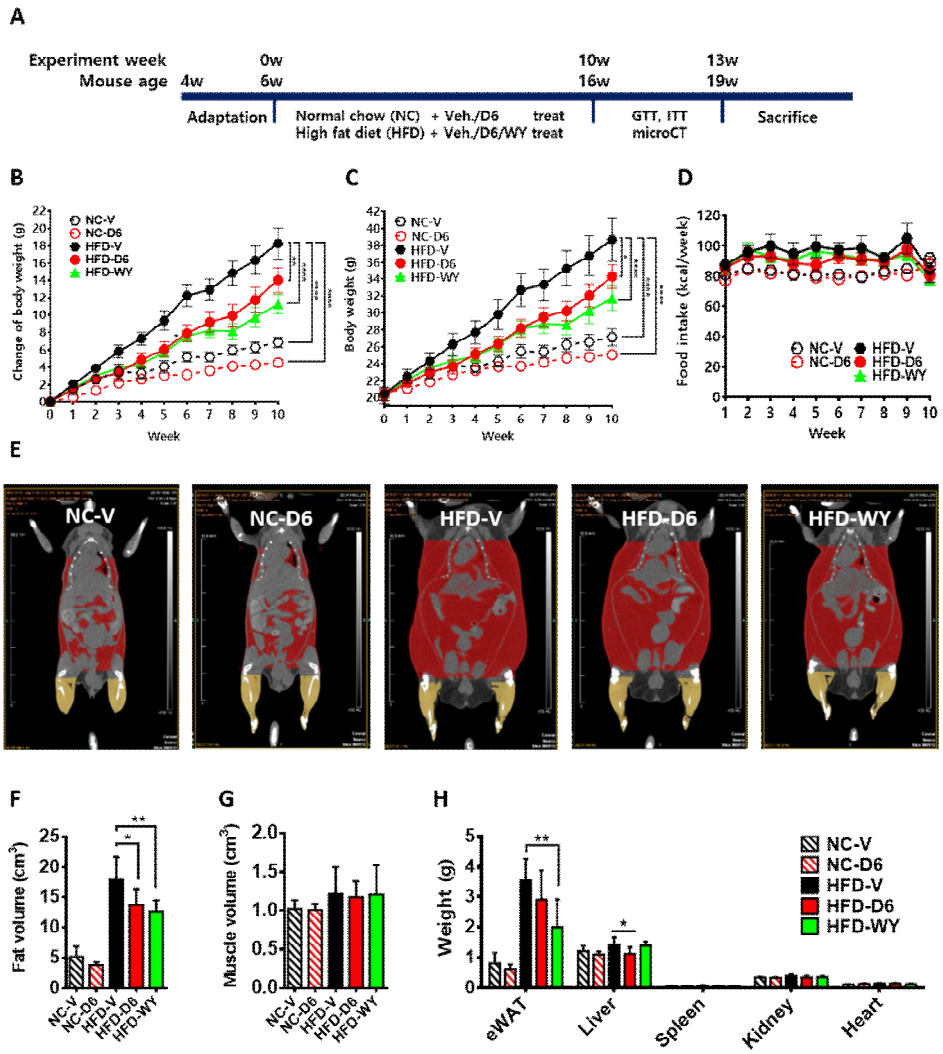


**Figure 2. D6 reduces the lipid droplet accumulated by rosiglitazone at 3T3-L1.**

(A) Representative photomicrographs of differentiated 3T3-L1 cells stained with Oil Red O (Scale bar 100μm, Magnification 200X). 3T3-L1 cells were treated with the drug during differentiation. DMSO (vehicle) was treated with 0.1% as the negative control. Ro means treating rosiglitazone, which causes differentiation and lipid accumulation. D6+Ro means the treatment of D6 and rosiglitazone together during differentiation. The concentration of drugs is 25μM. (B) Quantitative measurement of lipid accumulation in differentiated 3T3-L1 cells. Oil Red O dye was isolated with iso-propanol from the stained cells and measured by NanoQuant Magellan 7 at OD 492nm. Data represent the mean ± SEM and statistical significance was tested by one-way ANOVA followed by Dunnett's multiple comparisons test. (C) The distribution of lipid droplet size was measured by ImageJ. Data are expressed as mean ± SEM and statistical significance was tested by two-way ANOVA followed by Dunnett's multiple comparisons test. N=3 independent experiments. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001 (versus DMSO), $^{\#}$P ≤ 0.05, $^{\#\#}$P ≤ 0.01 (versus Rosiglitazone).

**Figure 3.**



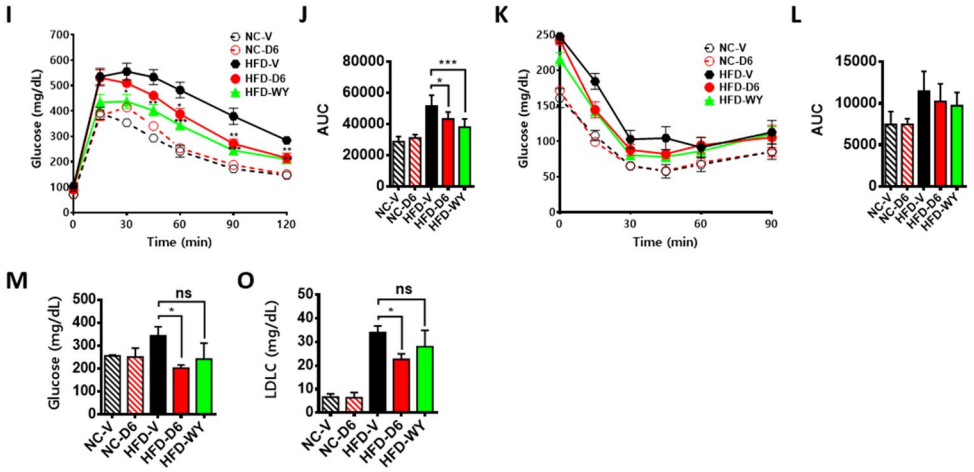
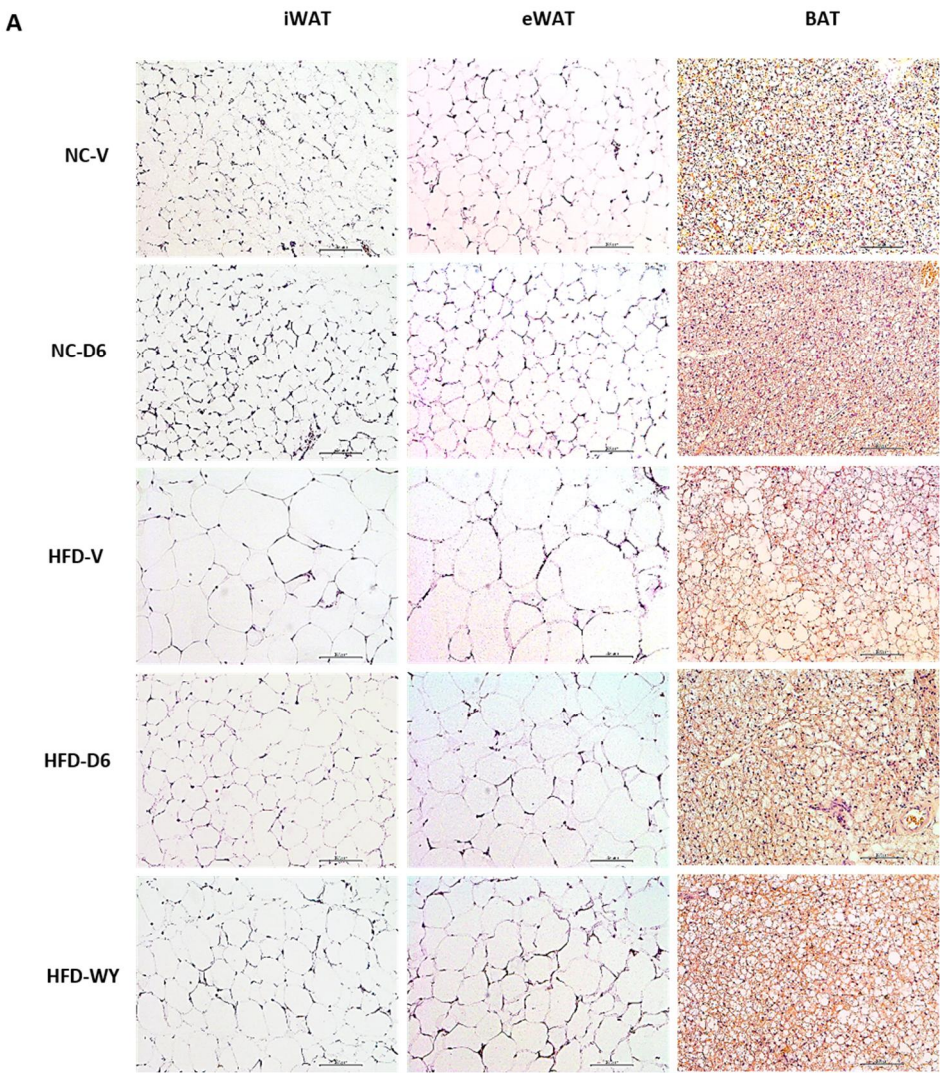



**Figure 3. D6 decreases body weight gain and relieves glucose metabolism in mice.**

(A) Mouse experimental schedule. After adaptation for 2 weeks, the mice were treated with a vehicle or 5 mg/kg/day of D6 or WY14643 by oral gavage for 13 weeks and induced obese by 60% HFD. After the treatment of drugs for 10 weeks, IP-GTT, IP-ITT, micro-CT were performed. (B) Change of body weight, (C) body weight and (D) food intake of mice observed for 10 weeks on an HFD. (E) Representative images of the micro-CT. Fat and muscle were shown in red and yellow, respectively. (F) Fat and (G) muscle were quantified with micro-CT image. (H) weight of organs (eWAT, liver, spleen, kidney, heart). (I) IP-GTT in mice after 10 weeks of HFD and (J) area under the curve (AUC) for this IP-GTT. (K) IP-ITT in mice after 12 weeks of HFD and (L) AUC for this IP-GTT. (M) Serum biochemical analysis of Glucose and (O) LDLC. Data are expressed as mean ± SEM and statistical significance was tested by one-way ANOVA followed by Dunnett's multiple comparisons test. N≥5 per group. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001 (versus NC-V)

**Figure 4.**



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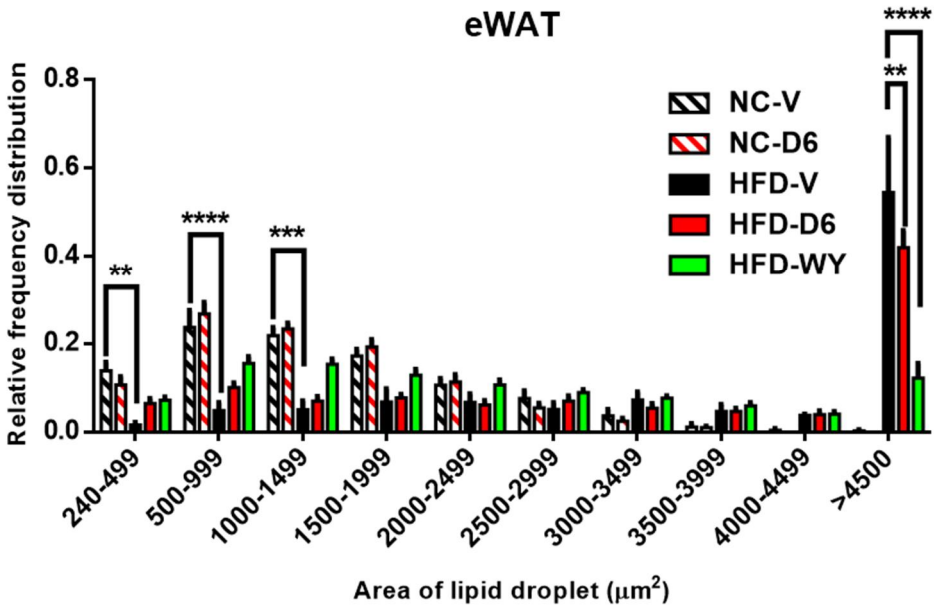



C

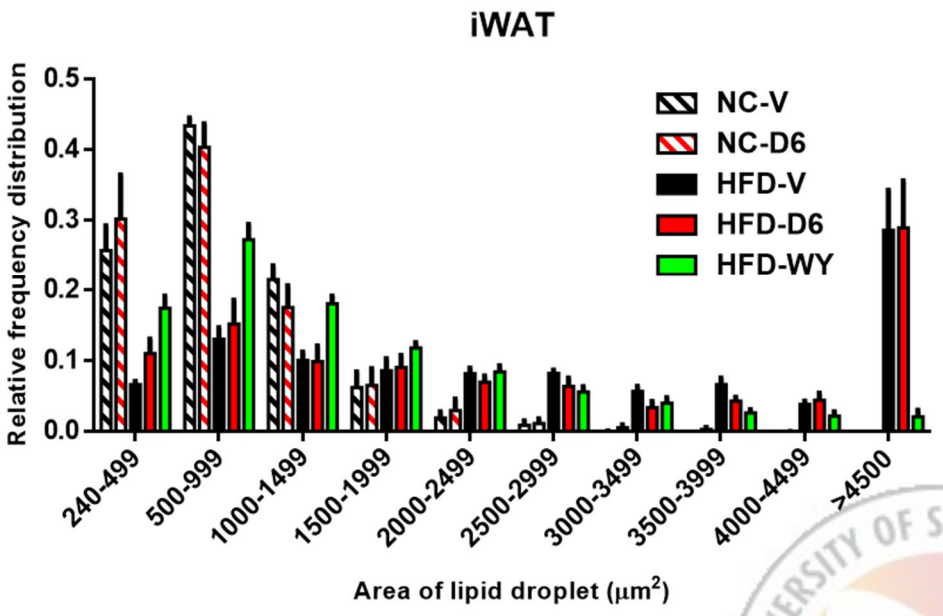
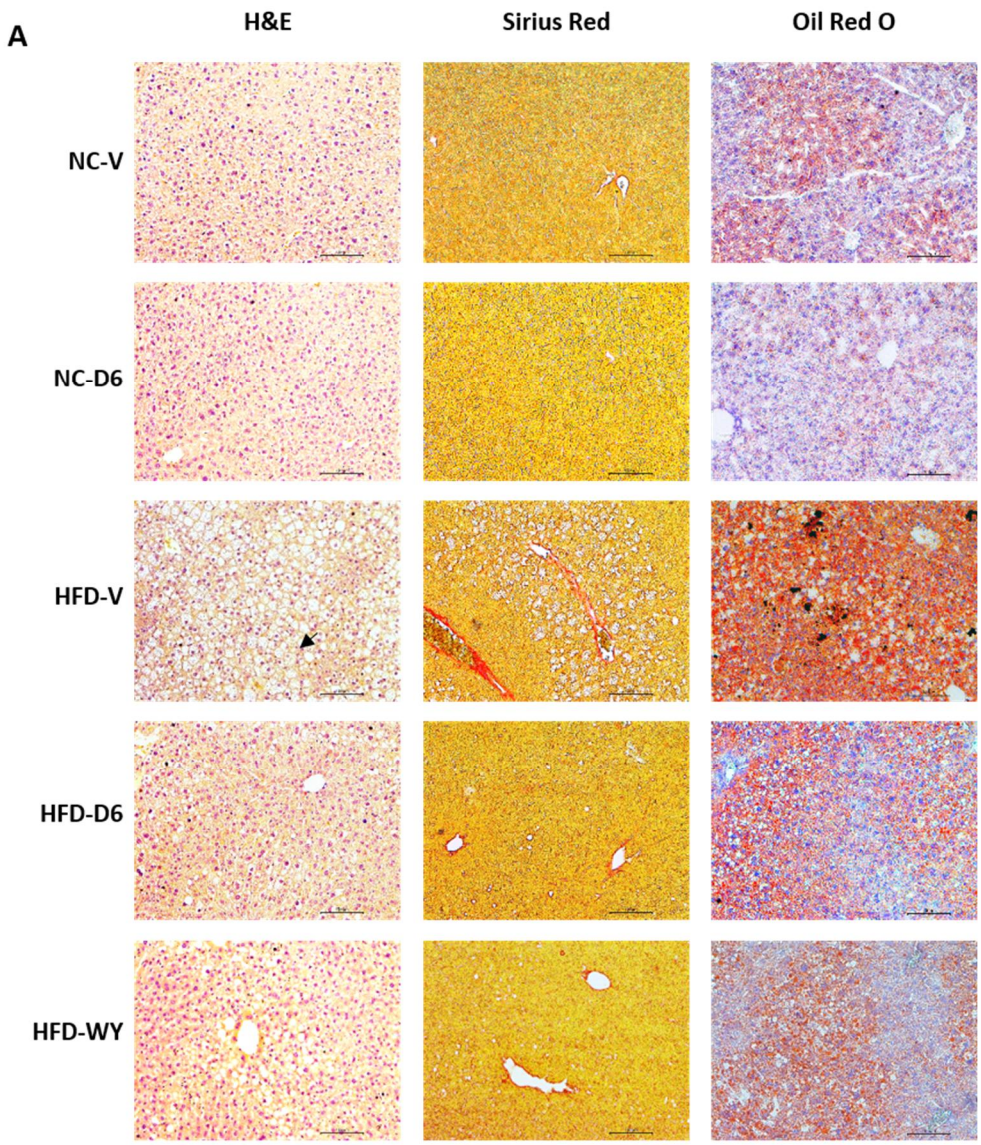


**Figure 4. Histological analysis of mouse adipose tissue.**

(A) Representative images of H&E stained sections of adipose tissues from mice fed HFD for 13 weeks. (Scale bar 100μm, Magnification 200X). (B) eWAT and (C) iWAT were quantified with H&E image. Data are expressed as mean ± SEM and statistical significance was tested by one-way ANOVA followed by Dunnett's multiple comparisons test. N≥5 per group. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001 (versus NC-V)

**Figure 5.**



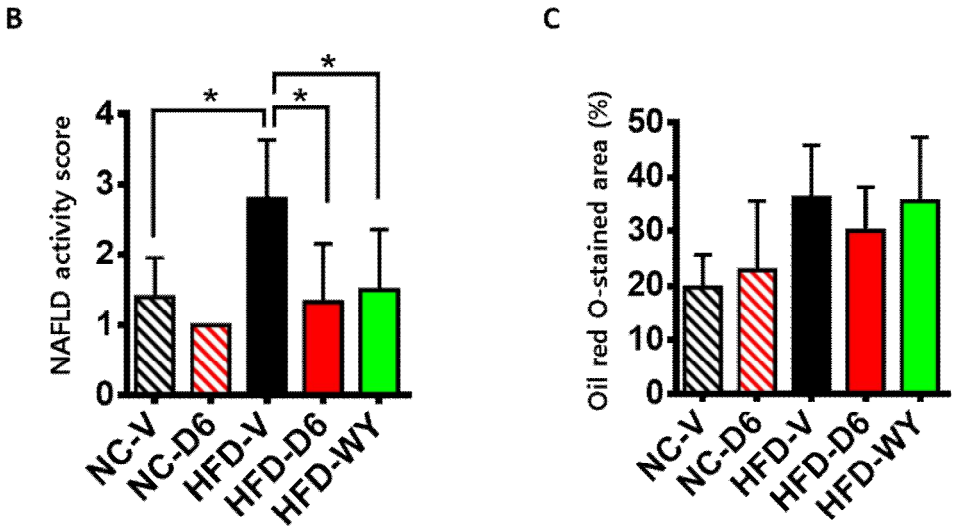



**Figure 5. Histological analysis of mouse liver.**

(A) Representative images of H&E, Oil Red O and Sirius Red stained sections of liver from mice fed HFD for 13 weeks. (Scale bar 100μm, Magnification 200X). An Arrow indicates ballooning. (B) NAFLD activity score and (C) Oil Red O-stained area were quantified with the H&E and Oil Red O of liver images, respectively. Data are expressed as mean ± SEM and statistical significance was tested by one-way ANOVA followed by Bonferroni's multiple comparisons test. N≥5 per group. *P ≤ 0.05, (versus NC-V) #P ≤ 0.05 (versus HFD-V). In Oil Red O-stained arear, P-value = 0.3377 (HFD-V vs HFD-D6)

## Ⅶ. TABLES

**Table 1. binding energy of PPARα and compounds.**

| Target of PPARα | Binding Energy of WY14643 | Binding Energy of D6 |
|---|---|---|
| 3vi8 | -7.8 | -8.3 |
| 5hyk | -6.9 | -8.4 |
| 4bcr_A | -7.6 | -8.3 |
| 4bcr_B | -7.6 | -8.6 |

**Table 2. binding energy of PPARδ and compounds.**

| Target of PPARδ | Binding Energy of GW501516 | Binding Energy of D6 |
|---|---|---|
| 5u3q_A | -11.4 | -9.4 |
| 5u46_A | -10.9 | -7.7 |
| 5u46_B | -10.7 | -9.3 |

**Table 3. binding energy of PPARγ and compounds.**

| Target of PPARγ | Binding Energy of Rosiglitazone | Binding Energy of D6 |
|---|---|---|
| 3u9q | -10.7 | -7.7 |
| 5ycp | -9.9 | -8.2 |
| 5ji0 | -10.7 | -8 |

# Ⅷ. SUMMARY IN KOREAN

비만은 전 세계적으로 증가하고 있습니다. 따라서, WHO는 1996년부터 비만을 질병으로 정의했습니다. 비만이 세계적으로 주목을 받는 이유는 비만이 여러 대사질환을 수반하고 개인뿐만 아니라 공공적으로 금전적 부담을 주기 때문입니다. 비만으로 야기되는 대사질환에는 당뇨, 고혈압, 심장병, NAFLD, 등이 있습니다. 대사질환의 근본적인 원인인 비만을 치료해서 비만으로 인한 여러 합병증 또한 예방하고자 합니다. 현재 사용되는 비만치료제는 CNS에 관여하여 식욕을 억제하기 때문에 여러 정신적 부작용을 일으킬 수 있습니다. 따라서 부작용을 최소화하고 항비만 효과가 뛰어난 새로운 약물이 필요한 실정입니다.

비만 치료제를 위한 타깃으로 PPAR이 많이 언급됩니다. 왜냐하면 PPAR은 전사인자(transcription factor, TF) 로 작용하고 전반적인 지질과 당의 물질대사를 조절하기 때문입니다. PPAR agonist는 동화 작용을 야기하지 않으며 안드로겐 수용체에 결합하지 않습니다. 그리고 PPAR agonist는 HPGA를 억제하거나 테스토스테론 생산에 영향을 미치지 않습니다. 마지막으로 PPAR agonist는 지구력을 증가시키지만 각성제 특성이 없으므로 수면 또는 코르티솔 수치에 영향을 미치지 않습니다. 이런 장점으로 많은 연구자는 PPAR agonist를 찾는 노력을 하고 있습니다. PPARα는 이상지질혈증에, PPARδ는 체중감소에 효과적입니다. PPARγ agonist는 주로 당뇨의 치료제로 사용되지만, 체중이 증가하는 부작용이 있습니다. 이에 PPAR dual, 혹은 pan agonist를 대사질환 치료제로 응용하기 위한 여러 시도가 진행되고 있습니다. Pan agonist의 장점은 전반적인 물질대사를 조절하는 것입니다. 대사질환의 경우 여러 원인이 복합적으로 작용하는 질병이기 때문에 한 종류의 PPAR을 활성화하는 selective agonist보다 모든 종류의 PPAR을 활성화하는 pan agonist를 이용하는 것이 대사질환을 효율적으로 치료할 수 있습니다.

이번 연구에서, 우리는 D6가 비만 치료제로써 가능성이 높은 PPAR pan-agonist라는 것을 처음으로 밝혔습니다. 그리고, D6가 비만뿐 아니라, 비만으로 유도된 대사질환 특히 NAFLD의 치료제로서 가능성이 높음을 시사하고 있습니다. 왜냐

하면 D6는 모든 종류의 PPAR에 결합하여 활성화하고 특히 PPARα를 우세하게 활성화하기 때문입니다.

또한, 그동안 Synthetic compound D6에 관한 보고가 전혀 없었습니다. D6의 세포 내에서 PPAR pan agonist 효능을 밝히고 D6는 비만을 억제하는 경향성을 mouse 실험을 통해 알게 되었습니다.

하지만, D6가 PPAR 다음으로 정확히 어떤 molecular pathway를 통해 세포 내에서 lipid and glucose metabolism을 활성화하는지 모릅니다. D6의 영향으로 물질대사에 관여하는 유전자의 변화를 알아보는 실험을 통하여 분자적 작용에 대한 연구가 필요합니다. 또한, 본격적인 임상시험을 통해 더 다양한 독성 검사를 통해 확인해야 합니다. 정리하면 독성 검사로 D6의 안전성 문제가 확실히 하고, 분자적 작용에 대한 연구로 D6의 작용 원리를 명확히 할 과제가 남아있습니다. 안전성과 작용기작이 명확해지면 D6는 대사질환의 새로운 치료제로서 주목을 받을 수 있기에 기대하고 있습니다.

# IX. ACKNOWLEDGEMENT

## 감사의 글

2017년 1월부터 약 3년의 시간 동안 MNP에 있으면서 저에게 학문적 지식 뿐 아니라 내면을 발전할 수 있는 시간이었고, 정말 감사한 경험이었습니다. 대학원에 와서 처음으로 제 자신과 진지하게 마주할 기회를 가질 수 있었습니다. 아직 부족한 점이 많지만 온전한 사람으로 발전하기 위해 지금처럼 꾸준히 노력하겠습니다. 그리하여 제가 받은 가르침과 도움을 주위 사람에게도 전하며 선한 영향력을 끼치는 사람이 되길 희망합니다. 학위 과정 동안 저에게 큰 가르침과 도움을 주신 모든 분들께 감사의 인사를 드립니다.

먼저, 제가 연구에만 전념할 수 있도록 전폭적인 지원을 해주신, 저의 지도교수님 김경태 교수님께 감사의 말씀을 올립니다. 2016년 가을에 김경태 교수님께 떨리는 마음으로 이메일을 보낸 인연을 시작으로 현재까지 교수님과 함께 지내면서 교수님의 연구에 대한 열정, 성실, 결단, 단호, 온화, 학생에 대한 애정 등을 바로 옆에서 지켜볼 수 있었습니다. 교수님처럼 닮기 위해 저 스스로 노력을 했지만, 아직 부족함이 많다는 것을 느낍니다. 처음에 제가 교수님의 기대에 미치지 못했기에 저에게 쓴 소리를 많이 하셨고, 그동안 걱정을 많이 하셨다는 사실을 잘 알고 있고 송구합니다. 항상 변함없이 노력하시는 교수님, 존경합니다. 치료제 후보를 찾고 그에 대한 연구가 많은 금전적, 시간적 자원이 많이 듭니다. 이런 지원을 교수님께서 아낌없이 주셨기에 아무런 걱정 없이 편하게 실험만 할 수 있었습니다. 진심으로 감사합니다.

또한, 신근유교수님께도 감사함을 전합니다. 고가의 장비를 사용함에 있어서 흔쾌히 허락해 주셔서 감사합니다. 덕분에 좋은 이미지 데이터를 얻을 수 있었습니다. 그리고 저와 마주치실 때마다 먼저 안부를 건내 주셔서 감사합니다. 우연

히 복도에서 만나 신근유 교수님과 대화할 수 있어서 즐거웠습니다.

더불어 학위논문 심사위원 김민성 교수님, 백승태 교수님께 감사함을 전합니다. 심사위원 교수님께서 해주신 귀한 말씀들을 통해 저의 연구를 더욱 성찰할 수 있었습니다. 앞으로도 여기서 배운 만큼 큰 책임감을 가지고 노력을 다하는 연구원이 되고자 노력하겠습니다.

효진선배. 실험실에서 묵직한 무게감을 주셨던 선배가 가시고, 선배가 많이 그리웠습니다. 건강하게 딸을 출산하신 것 축하합니다. 같이 지낸 시간은 짧았지만 그동안 저를 막내라고 챙겨주셔서 감사합니다.

도현선배. 팀이 바뀌고, 좀 친해지려 했는데 바로 졸업하셔서 개인적으로 아쉬웠습니다. 그래도 근처에 계셔서 무슨 일 있을 때마다 도현선배에게 연락하고 많이 의지했습니다. 친절하게 알려주셔서 그동안 감사했습니다.

정현언니. 제가 처음 포항에 와서 아무것도 모르던 시절 저에게 가장 많이 쓴 소리를 해주신 정현언니에게 항상 감사합니다. 정말 애정 어린 쓴 소리를 한다는 것이 칭찬보다 더 어렵다는 것을 최근에서야 깨닫고 있습니다. 그 쓴 소리가 처음엔 많이 아팠지만, 모두 다 피가 되고 살이 되는 조언이었습니다. 지금에도 실험을 할 때면 언니가 해주신 조언들이 되새기고, 그 조언대로 더욱 조심하게 실험에 임하려 노력합니다. 그리고 언니와 밤새 이야기했던 작년 이맘 때가 그립습니다. 부단한 노력으로 제가 더 성숙해지고, 발전해서 언니가 다시 한국 오시는 날, 언니의 기대에 부흥할 수 있기를 바랍니다.

지영언니. 문제가 될 수 있는 저의 행실에 대해 조언을 해주셔서 감사했습니다.

영섭오빠. 많은 접점은 없었지만, 여쭤보았을 때, 친절히 알려주셔서 감사합니다.

영훈오빠. 오빠 뒷자리로 자리를 옮기면서 더 많이 같이 디스커션하고 즐거웠습니다. Protein 같이 뽑아 주셔서 정말 감사합니다. 덕분에 수월하게 실험할 수 있었습니다.

주현언니. 졸업 준비로 정신 없는 와중에 마지막까지 OCT block 만들고,

cryosection 실험을 친절히 알려주셔서 감사합니다.

혜국선배. Biotinylation 실험과 neuclear-cytoplasm fraction을 선배에게 배울 수 있어서 좋았습니다. 선배와 같이 duplicate로 실험을 배웠던 기억이 좋아서 저도 후배에게 알려줄 때 시간과 노력이 더 들더라도 같이 duplicate로 실험을 진행하고자 노력합니다. 그동안 챙겨주셔서 감사했습니다.

광호오빠. 랩의 분위기를 좋게 유지하기 위해 노력해 주셔서 감사합니다. 실험적으로, 생활적으로 어떤 문제가 생겼을 때, 오빠에게 바로 물어볼 수 있어서 감사했습니다. 또한, 디스커션을 부탁했을 때, 주저없이 바로 시간 내주시고 상세하게 설명해주셔서 감사합니다.

승현선배. 제가 사회 생활이 전무했기에 처음에 튀는 행동을 많이 했는데, 그 때 승현선배께서 바로 쓴 소리 해주신 덕분에 빨리 고치고 배울 수 있었습니다. 바로 쓴 소리 해주셔서 진심으로 감사했습니다. 그리고 그동안 선배 실험에 지장을 주고 신경 쓰이게 해드려서 죄송합니다.

은지언니. 제가 적응하는데 많은 도움을 주시고, 같이 수업 듣고, 미국 워싱턴으로 언니와 같이 학회 갈 수 있어서 좋았습니다. 은지언니가 해주신 조언 잊지 않고 더 발표를 매끄럽게 할 수 있도록 노력하겠습니다. 그동안 감사했습니다.

원식. 같이 comp.cell 만들고 in silico 및 여러가지로 많이 도와줘서 고마워.

성욱씨. 제가 차량 부탁했을 때 흔쾌히 매번 들어주셔서 그동안 감사했습니다.

경원씨. 이야기는 많이 못했지만, 제 요구를 잘 수용해 주셔서 감사했습니다.

종수오빠. 제가 잘 몰라서, 대사질환 관련 연구를 하는 오빠에게 참 많이 여쭤보았는데, 그 때마다 친절하게 답변해주셔서 정말 감사했습니다.

성지씨. 정말 수고 많으셨어요. 여름에 처음 오자마자 microCT를 찍었는데, 마지막에도 microCT를 찍고 끝나네요. 짧았지만 여러 실험을 가장 많이 저와 같이 해주셔서 정말 감사했습니다.

현주씨. 친해질 계기가 없어서 많은 이야기를 하지는 못했지만, 지금 하시는 연구 잘 하셔서 좋은 성과가 있기를 바랍니다.

승희씨. 말수가 적고, 내색하지 않아서 쓰러질 정도로 힘드셨는지 몰랐어요. 바쁘다는 핑계로 더 챙겨드리지 못해 죄송하고, 건강 잘 챙기셔서 하시는 연구 좋은 성과 있기를 바랍니다.

주연언니. 언니와 함께한 모든 추억이 정말 소중해요. 저와 같이 시간 보내주셔서 감사합니다. 언니와 같이 이야기했던 모든 내용이 저에게 큰 힘이 되고, 그동안 저의 이야기를 들어주시고, 조언해 주셔서 정말 고맙습니다.

지혜어머님. 어머니께서 매일 아침에 반갑게 환한 미소로 인사해 주셔서 감사합니다. 제가 실험하다가 마음이 급해서 실수로 엎지르게 되면 꼭 같이 뒷정리를 해주시고 정말 감사합니다.

지경영선생님. 같이 사진 찍으면서 많은 이야기했던 추억이 오래 남을 것 같습니다. 편히 실험할 수 있도록 배려해주셔서 진심으로 감사드립니다. 지경영 선생님 덕분에 새벽에 나오면서도 즐겁게 실험할 수 있었습니다.

김영기선생님. 동물실험실을 관리 해주셔서 감사합니다. 덕분에 동물 실험하는데, 편하게 할 수 있었습니다.

강성아박사님. TP로 microCT를 찍으러 갈 때마다 친절하게 연락 받아주셔서 진심으로 감사드립니다. 강성아박사님 덕분에 동물을 최우선으로 배려하며 실험해서 좋은 결과 얻을 수 있었습니다. 감사합니다.

임지환선생님. 총 마우스의 수도 많고, 보아야하는 장기도 많아서 샘플이 100개 이상이었는데 최대한 빠르게 실험해 주셔서 진심으로 감사드립니다.

부모님께, 아버지. 어머니. 제가 대학을 서울로 갈 수 있었던 것도, 대학원을 포항까지 올 수 있었던 것도 두 분의 희생과 사랑이 있으셨기 때문입니다. 그 희생과 사랑이 없었다면 지금의 학위는 불가능했을 것입니다. 제가 하고 싶은 일의 대부분을 이루어 주시기 위해 최선을 다하시는 부모님께 항상 감사하고 고맙

고 사랑합니다. 언제나 건강하시고, 행복하길 바랍니다.

재영아. 내가 집에서 떨어진 지 8년이 넘었지만 그동안 집에 걱정을 덜 할 수 있었던 이유는 네가 집에 있었기 때문이야. 내년에는 네가 울산으로 가서 독립하지만, 울산에서도 네가 원하는 삶을 찾길 바랄게.

혜화야. 너는 정말 우리 가족의 소중하고 사랑스런 막내야. 이 사실을 잊지 않고 행복하게 네가 원하는 꿈 이루며 살 수 있길 바라.

아버지, 어머니와 더불어 공순고모, 영숙이모께서 자랑스러워하는 모습을 보기 위해 힘들어도 참고 공부하다 보니 어느덧 대학원까지 오게 되었습니다. 제가 다시 힘내서 공부할 수 있게 지칠 때마다 격려와 응원해주신 공순고모, 영숙이모께도 감사의 말씀 드립니다.

언니들이 있어 든든해. 고은언니. 매번 내가 비빌 언덕이 되어주어서 고마워. 언니와 같이 2016년도 여름에 대학원 입시를 준비했는데, 이번 대학원 논문도 같이 마무리 도와줘서 정말 고마워. 예선언니, 경화언니. 우리 언니들 덕분에 전주에 가족들 상황을 바로 알고 나 대신 우리집에서 딸 노릇, 언니 노릇 해줘서 정말 고마워.

같이 왔지만, 카이스트로 간 지현언니. 진솔이, 우성오빠, 무형오빠, 소영이. 지현언니 덕분에 대학교 때 즐거웠어. 매일 같이 점심, 저녁 먹고 카페에서, 도서관에서 공부했던 일, 기말 끝나고 놀러갔던 일, 정말 추억이 많아. 그동안 나와 함께 해줘서 고마워. 앞으로도 좋은 추억 많이 만들자.

대학부터 같이 대학원까지 온 혜리야. 네가 없었다면 정말 난 정말 외롭고 중간에 포기했을 거야. 네가 있었기에 마무리를 할 수 있었어. 내 옆에 있어줘서 고마워. 나도 네게 힘이 될 수 있길 바라. 남은 학위 과정 꼭 좋은 성과 있기를 기도할게.

아름언니. 여름 인턴 때 처음 봤는데 벌써 내가 졸업이네. 그동안 언니도 랩에 적응하고 연구하느라 힘들었을 텐데... 나까지 챙겨줘서 정말 고마워. 언니와 함께한 모든 시간 잊지 못할 거야. 언니가 있어서 고마웠어.

유빈언니. 바빠서 자주 보진 못했지만, 볼 때마다 밝게 인사 받아줘서 고마워. 졸업 축하해.

은지야. 실험적으로 많이 부탁을 해서 귀찮을 텐데도 싫은 내색없이 도와줘서 고마워. 네가 실험실 분들과 나 사이에서 중간 다리를 잘 해줬기 때문에 아무런 잡음 없이 이미지 데이터를 얻을 수 있었어. 정말 고마워.

가은자매님, 아인자매님, 저의 일을 자신의 일처럼 여겨 주셔서 감사합니다. 설화자매님, 아라자매님, 혜인자매님, 청년회 형제자매님, 장재영집사님, 윤봉녀자매님, 나상법 목사님, 서만석전도사님, 그 밖에 저에게 먼저 따뜻하게 맞아 주신 포항교회 식구들. 모두 감사합니다.

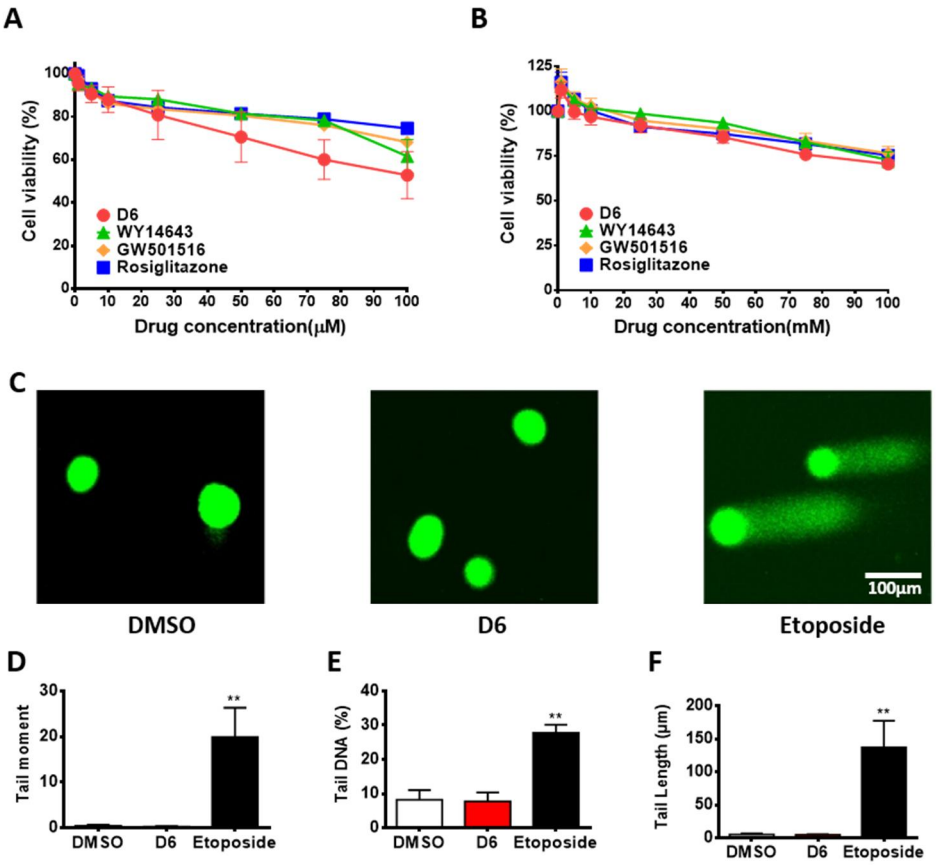
마지막으로 하나님. 하나님께서 저를 보시면 항상 기쁘시기를 바랍니다. 제 마음, 말, 행동 모두 하나님께서 지켜주시고, 주의 선하신 뜻대로 제 인생의 길을 이끌어 주시길 바랍니다.

이름을 다 적지 못했지만, 지금의 제가 있기까지 저를 도와주신 분들 정말 진심으로 감사드립니다. 앞으로 주위 사람에게 받은 도움을 드릴 수 있는 사람으로 발전하기 위해 부단히 노력하겠습니다. 감사합니다.

# Ⅷ. APPENDIX

## LIST OF FIGURES

**Figure S1.**



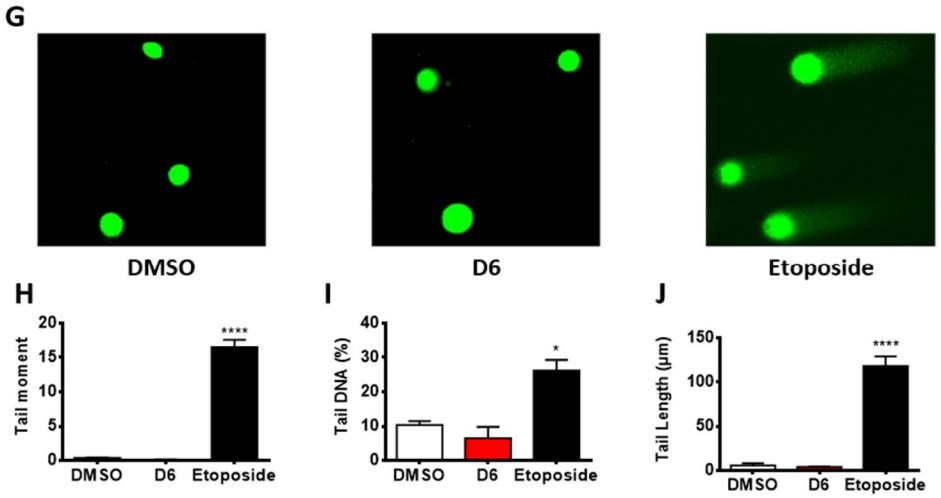



**Figure S1. D6 has low cytotoxicity in major experiment concentration in HEK293 and 3T3-L1.**

(A) MTT assay of HEK293 treated with different concentrations of drugs. (B) MTT assay of 3T3-L1 treated with different concentrations of drugs. N=3-4 independent experiments. (C) Representative images of Comet assay in HEK293 and (D-F) analysis of the image by CometScore 2.0. (Scale bar 100μm). The concentration of drugs is 50μM. (G) Representative images of Comet assay in 3T3-L1 and (H-J) analysis of the image by CometScore 2.0. (scale bar same). The concentration of drugs is 50μM. Data shown represent the mean ± SEM and statistical significance was tested by one-way ANOVA followed by Dunnett's multiple comparisons test. N=3-4 independent experiments. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001 (versus DMSO)

**Figure S2.**

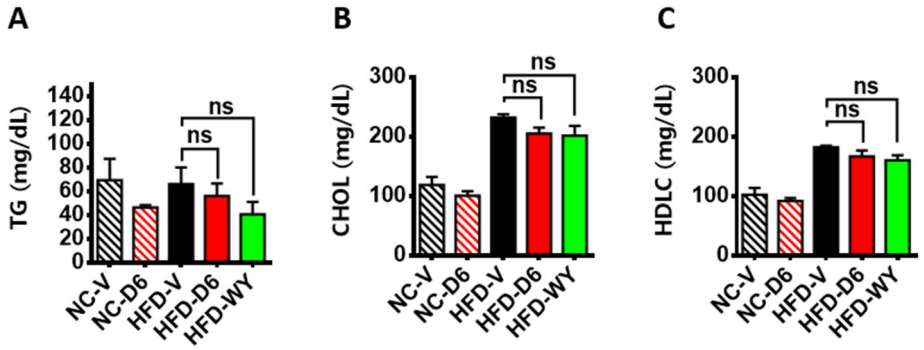



**Figure S2. Serum biochemical analysis about lipid.**

(A) Serum biochemical analysis of triglyceride (TG), (B) cholesterol (CHOL), and (C) high-density lipoprotein cholesterol (HDLC). Data are expressed as mean ± SEM and statistical significance was tested by one-way ANOVA followed by Dunnett's multiple comparisons test. N≥5 per group (Not significant, ns).

**Figure S3.**

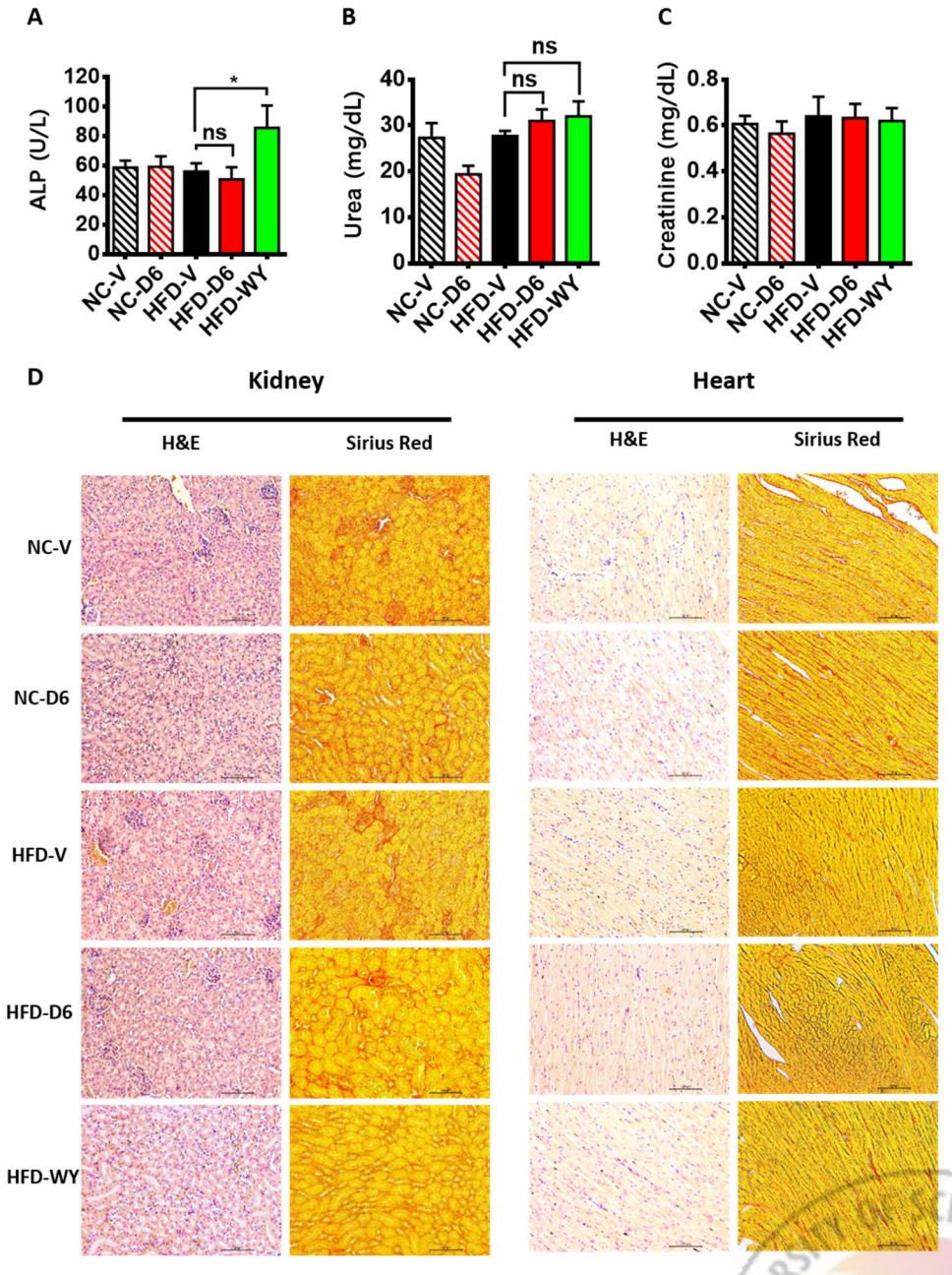


**Figure S3. There is little difference in phenotypes between vehicle and D6-treatment group in mouse other tissue.**

(A) Serum biochemical analysis of ALP, a marker of liver damage. (B) Serum biochemical analysis of urea, and (C) creatinine, markers of kidneys disease progresses. Data are expressed as mean ± SEM and statistical significance was tested by one-way ANOVA followed by Dunnett's multiple comparisons test. N≥5 per group (*P ≤ 0.05, Not significant, ns). (D) Representative H&E and Sirius Red images of histological analysis on different organs, kidneys and heart for the identification of organ toxicity (Scale bar 100μm, Magnification 200X). N≥5 per group.

**Figure S4.**

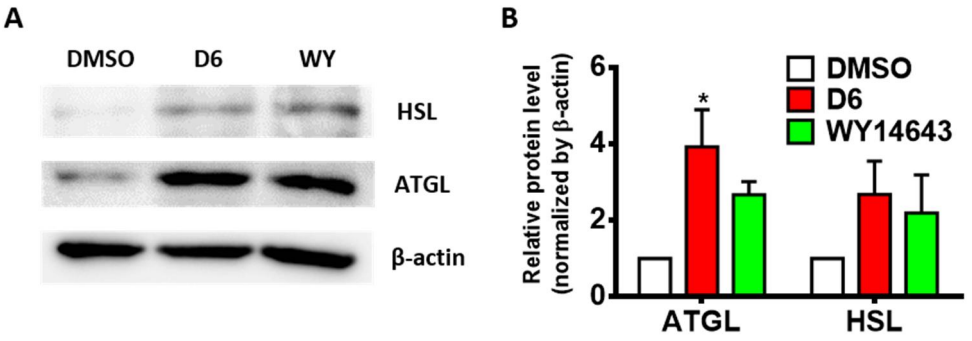



**Figure S4. D6 regulates gene expression related to lipid metabolism in 3T3-L1.**

(A) Representative Western blots for HSL, ATGL in mature 3T3-L1 treated with D6 and WY14643 during differentiation. The concentration of drugs is 50μM. (B) Quantitative measurement of band intensity by ImageJ and normalized to β-actin. Data are expressed as mean ± SEM and statistical significance was tested by one-way ANOVA followed by Dunnett's multiple comparisons test. N=3 independent experiments. *P ≤ 0.05, (versus DMSO).

**본 학위논문 내용에 관하여 학술, 교육 목적으로 사용할**

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