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# Metformin enhances the anti-adipogenic effects of atorvastatin via modulation of STAT3 and TGF-β/Smad3 signaling

Byung-Hak Kim [a,b], Songhee Han [a], Haeri Lee [a], Chi Hye Park [c], Young Mee Chung [c], Kyungmin Shin [c], Hyun Gyu Lee [d], Sang-Kyu Ye [a,e,f,*]

[a] Department of Pharmacology & Biomedical Sciences, Seoul National University College of Medicine, Seoul 110-799, Republic of Korea
[b] Biomedical Science Project (BK21[PLUS]), Seoul National University College of Medicine, Seoul 110-799, Republic of Korea
[c] R&D Center, CJ HealthCare Corp., Gyeonggi-do 467-812, Republic of Korea
[d] Department of Microbiology and Immunology, Yonsei University College of Medicine, Seoul, Republic of Korea
[e] Ischemic/Hypoxic Disease Institute, Seoul National University College of Medicine, Seoul 110-799, Republic of Korea
[f] Neuro-Immune Information Storage Network Research Center, Seoul National University College of Medicine, Seoul 110-799, Republic of Korea

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ABSTRACT

Adipocyte accumulation is associated with the development of obesity and obesity-related diseases. Interactions of master transcription factors and signaling cascades are required for adipogenesis. Regulation of excessive adipogenic processes may be an attractive therapeutic for treatment of obesity and obesity-related diseases. In this study, we found that atorvastatin exerts an anti-adipogenic activity in 3T3-L1 pre-adipocytes, and that this activity is elevated in combination with metformin. Expression of the adipogenic master regulators PPARγ and C/EBPα, and their target gene aP2, was suppressed by atorvastatin. Furthermore, atorvastatin treatment resulted in increased activation of the key master regulator of cellular energy homeostasis, AMPK. These biological activities of atorvastatin were elevated in combination with metformin. These anti-adipogenic activities were associated with regulation of the STAT3 and TGF-β signaling cascades, resulting in the regulation of the expression of STAT3 target genes, such as KLF5, p53, and cyclin D1, and TGF-β signaling inhibitory genes, such as SMAD7. Our results suggest that combination therapy with atorvastatin and metformin may have therapeutic potential for the treatment of obesity and obesity-related diseases caused by excessive adipogenesis.



## 1. Introduction

Adipose tissue, which is composed mainly of adipocytes, plays a key role in energy homeostasis by regulating lipid metabolism, and is recognized as a major endocrine organ [1]. Excessive accumulation of adipocytes leads to obesity and obesity-related diseases, including diabetes, hyperlipidemia, hypertension, arteriosclerosis, fatty liver, and cardiovascular diseases [2]. Adipogenesis, a complex process involving transcription factors and signaling cascades, is implicated in the excessive accumulation of body fat. It leads to coordinated changes in cellular morphology, hormone sensitivity, signaling pathways, and transcriptional regulation [3]. A number of transcription factors, including peroxisome proliferator-activated receptor gamma (PPARγ) and the CCAATT enhancer binding protein (C/EBP) family, are required for adipogenic differentiation [3,4]. Recently, signal transducer and activator of transcription 3 (STAT3) was demonstrated to play a key role in adipogenesis through PPAR-dependent signaling [5,6], highlighting the importance of regulation of PPAR-STAT3 signaling in the context of high-fat-associated human diseases.

Statins were primarily developed as inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, and have been used clinically for the treatment of dyslipidemia [7]. Beyond their cholesterol-reducing activities, statins have been reported to be beneficial for treatment of many other conditions, including dementia, kidney disease, cancer and inflammatory diseases [8–12]. Metformin is the most-prescribed drug for type 2 diabetes [13], and has also been found to have efficacy in the treatment of premature puberty, cancer, and non-alcoholic fatty liver disease [14–16]. In addition, this reagent is prescribed for anti-diabetic therapy, in combination with anti-hypertensive agents, including atorvastatin [17,18]. A growing number of reports demonstrate that statins and metformin increase the anti-cancer, anti-angiogenesis, and

* Corresponding author at: Department of Pharmacology and Biomedical Sciences, Seoul National University College of Medicine, 103 Daehak-ro, Jongno-gu, Seoul 110-799, Republic of Korea.
*E-mail address:* sangkyu@snu.ac.kr (S.-K. Ye).

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cardiac-function-improving effects of common agents such as cisplatin, bevacizumab, and evacetrapib [19–21]. This evidence suggests that combination therapy with statins and metformin may be an effective treatment strategy for several human diseases. In this study, we demonstrate that atorvastatin exerts an anti-adipogenic effect, and that this effect is elevated when administered in combination with metformin. We further determine the molecular mechanisms underlying the inhibition of adipocyte differentiation by these drugs.

## 2. Materials and methods

### 2.1. Reagents

Atorvastatin and metformin hydrochloride were kindly provided from the Reddy's Laboratories and the Harman Finochem (India), respectively. Recombinant human IL-6 and TGF-β1 were obtained from R&D systems (Minneapolis, MN, USA). Dexamethasone, isobutyl-1-methylxanthine (IBMX) and insulin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibodies specific for phospho-STAT3 (Tyr705), STAT3, phospho-AMPK (Thr172), AMPK, PPARγ, C/EBPα, Smad3, and GAPDH were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-phospho-Smad3 (Ser423/425) antibody was obtained from Epitomics (Burlingame, CA, USA).

### 2.2. Cell culture

Murine 3T3-L1 pre-adipocytes (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin solution (Gibco, Grand Island, NY, USA). For adipocyte differentiation, cells were seeded at a density of $5 \times 10^4$ cells/ml in 6-well tissue culture plates, and confluent cells were incubated for 2 days in DMEM containing 10% FBS and MDI (1 μM dexamethasone, 0.5 mM IBMX, and 5 μg/ml insulin). Cells were replaced with DMEM containing 10% FBS and 5 μg/ml insulin every 2 days. Post-confluent 3T3-L1 pre-adipocytes for 2 days were stimulated with the differentiation medium in the presence or absence of atorvastatin and/or metformin for 3 days to examine the effect adipocyte differentiation. The medium was then replaced with the maintenance media in the presence or absence of the indicated concentrations of atorvastatin and/or metformin every 3 days until the end of the experiment at day 9.

### 2.3. Cell viability assay

Cells were seeded at $1.0 \times 10^4$ cells per well in 96-well plates and incubated in culture medium until 70–80% confluence. The cells were treated with various concentrations of atorvastatin and metformin for 24–72 h. Cell viability was measured using a microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 450 nm after reaction for 2–4 h at 37 °C, followed by addition with the EZ-CyTox Enhanced Cell Viability Assay Reagent (Daeil Lab Service, Seoul, Korea), as previously described [22].

### 2.4. Western blot analysis

Cell lysates were resolved on SDS-PAGE and transferred onto nitrocellulose membranes (Sigma–Aldrich). The membranes were blocked in a blocking buffer and incubated with specific primary antibodies for the target molecules overnight at 4 °C. Blots were washed and incubated with horseradish peroxidase-conjugated secondary antibodies, and signals were detected using an ECL detection kit (SurModics, Inc, Eden Prairie, MN, USA).

### 2.5. Quantitative real-time PCR

RNA isolation and quantitative real time-PCR were conducted as described earlier [23], and the results were normalized to the signals of GAPDH expression. The following primers were used: STAT3 (F, 5′-GCCACGTTGGTGTTTCATAATC-3′ and R, 5′-TTCGAAGGTTGTGCTGATAGAG-3′), PPARγ (F, 5′-CATTCTGGCCCACCAAC-3′ and R, 5′-ATGCGAGTGGTCTTCCATCA-3′), C/EBPα (F, 5′-AGCAACGAGTACCGGGTACG-3′ and R, 5′-TGTTTGGCTTTATCTCGGCTC-3′), aP2 (F, 5′-CACCGCAGACGACAGGAAG-3′ and R, 5′-GCACCTGCACCAGGGC-3′), and KFL5 (F, 5′-TGAACGTCTTCCTCCCTGAC3-3′ and R, 5′-GGTCTGGTGGGAGCTGAATA-3′). The primers against p53, cyclin D1, and GAPDH were obtained from Qiagen (Valencia, CA, USA).

### 2.6. Statistics

Data were presented as the means ± standard deviation (SD) from three independent experiments. Differences were determined by the unpaired Student's *t*-test. Statistical significance was defined as *p*-value less than 0.05.

## 3. Results

### 3.1. Combination treatment with atorvastatin and metformin does not elevate cytotoxicity

Atorvastatin and metformin are two of the most-prescribed drugs for dyslipidemia and type 2 diabetes worldwide. Numerous pharmacological activities have been reported in the context of monotherapy or combination therapy with other reagents. We first determined their cytotoxic activity in 3T3-L1 pre-adipocytes by incubation with various concentrations of atorvastatin, metformin or a combination of the two reagents. These drugs exhibited weak-to-no cytotoxic activity over a 72-h period of monotherapy, at concentrations of up to 30 μM atorvastatin and 100 mM metformin. Combination therapy with various concentrations of atorvastatin and 100 mM metformin resulted in cytotoxicity similar to that of atorvastatin alone (Fig. 1A). Therefore, we used atorvastatin at concentrations of up to 20 μM, and metformin at 100 mM, to minimize the possibility of cytotoxic effects.

### 3.2. Inhibition of adipocyte differentiation is increased when atorvastatin and metformin are combined

Control of energy homeostasis is important for the prevention of metabolic diseases. AMP-activated protein kinase (AMPK) is a key master regulator of cellular energy homeostasis. Atorvastatin increased AMPK activation in a concentration-dependent manner and this activation was marginally enhanced in combination with metformin (Fig. 1B). However, the pan-JAK/STAT3 inhibitor AG-490 did not influence this activation. Adipocyte differentiation is regulated by the master regulators PPARγ and C/EBPα, which regulate target genes involved in pre-adipocyte differentiation into adipocytes. To investigate the effects of atorvastatin and metformin on adipocyte differentiation, we measured the levels of PPARγ and C/EBPα in 3T3-L1 cells. The mRNA and protein levels of PPARγ and C/EBPα were significantly suppressed by atorvastatin in a concentration-dependent manner and these effects were enhanced in combination with metformin (Fig. 1B and C). Consistent with these results, the mRNA levels of adipocyte fatty-acid-binding protein (aP2), a target gene of PPARγ and C/EBPα, were also suppressed by atorvastatin, and the inhibitory effects were again enhanced in combination with metformin (Fig. 1C). These data indicate that the anti-adipogenic activities of atorvastatin are synergized by combination therapy with metformin.

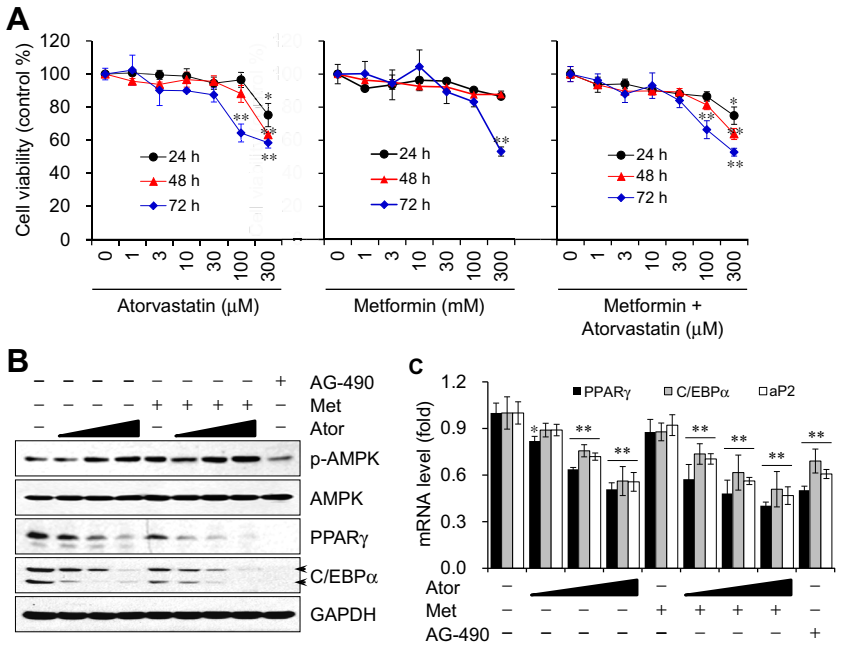


**Fig. 1.** Atorvastatin-induced adipocyte differentiation is enhanced when combined with metformin. (A) 3T3-L1 cells were incubated with various concentrations of atorvastatin (left), metformin (middle), or atorvastatin and metformin (100 mM, right), for 24–72 h. Cell viability was measured using the WST-1 reagent. (B and C) 3T3-L1 cells were treated with atorvastatin (Ator, 1–20 μM) and/or metformin (Met, 100 mM) for 5 days. Activation of AMPK and expression of adipogenic genes were assessed by Western blot analysis (B) and quantitative real-time PCR (C), respectively. Results are expressed as the means ± SD of three independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.005$. AG-490 (50 μM) was used as a pan-JAK/STAT3 inhibitor.

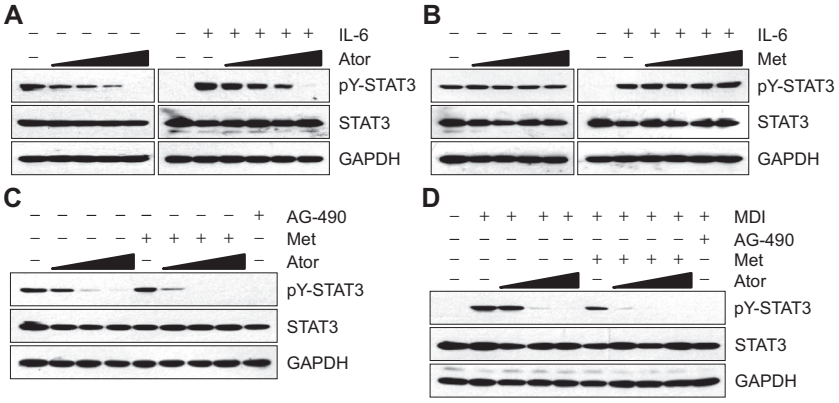


**Fig. 2.** Atorvastatin-induced inhibition of STAT3 activation is enhanced when combined with metformin. 3T3-L1 cells were treated with atorvastatin (1–20 μM, A), metformin (3-100 mM, B), or atorvastatin (1–20 μM) and metformin (100 mM, C and D), for 1 h, and then further incubated for 15 min in the presence or absence of IL-6 (10 ng/mL), or 14 h in the presence or absence of MDI. Western blot analysis was performed, with GAPDH serving as a loading control. Data are representative of one of three independent experiments with similar results. AG-490 (50 μM) was used as a pan-JAK/STAT3 inhibitor.

### *3.3. Inhibition of STAT3 activation is enhanced when atorvastatin and metformin are combined*

Recent reports suggest that STAT3 signaling is associated with adipogenesis [5,6], and that this signaling cascade in hepatocytes is inhibited by statins [24,25], suggesting that the anti-adipogenic activity of atorvastatin and metformin may be due to inhibition of STAT3 signaling. To address this possibility, we investigated the inhibitory effects of atorvastatin and metformin on STAT3 activation. Atorvastatin, but not metformin, inhibited tyrosine phosphorylation of STAT3 in a concentration-dependent manner, in the presence or absence of IL-6 in 3T3-L1 cells (Fig. 2A and B). The lack of an inhibitory effect of metformin on STAT3 activation is consistent with a previous report [26]. The inhibition of atorvastatin on STAT3 activation was enhanced in the presence of metformin (Fig. 2C). In addition, MDI-stimulated STAT3 activation was also inhibited by atorvastatin and these inhibitory activities were enhanced in combination with metformin (Fig. 2D and Supplementary Fig. S1). These results indicate that although metformin does not affect STAT3 activation in pre-adipocytes, combination therapy with atorvastatin and metformin may be a promising approach for inhibition of STAT3 signaling during adipogenesis.

### *3.4. Targeting STAT3 signaling exerts anti-adipogenic effects*

Our experimental data led us to investigate whether the anti-adipogenic effects of atorvastatin in combination with metformin are associated with inhibition of STAT3 signaling. To address this

possibility, we overexpressed or silenced STAT3 expression by transfecting 3T3-L1 cells with a wild-type (wt) or constitutively active (ca) STAT3 expression vector, or STAT3-specific siRNA. Activation of STAT3 signaling using wtSTAT3 or caSTAT3 has been shown to suppress AMPK activation and induces the expression of PPARγ, C/EBPα and aP2. However, inhibition of STAT3 signaling by combinational treatment with atorvastatin and metformin increased AMPK activation and suppressed the levels of PPARγ, C/EBPα and aP2 (Fig. 3A and B). Consistent results were observed following STAT3-specific siRNA-mediated STAT3 knockdown, which increased AMPK activation and suppressed the levels of PPARγ, C/EBPα and aP2 (Fig. 3A and B). These results clearly indicate that STAT3 signaling is important for adipogenesis, and that targeting STAT3 signaling with atorvastatin and metformin results in potent anti-adipogenic effects in 3T3-L1 cells.

### 3.5. Inhibiting STAT3 signaling with atorvastatin and metformin enhances STAT3 target gene expression

STAT3 signaling is known to regulate wide a range of target genes, including Krüppel-like factor 5 (KLF5), p53, and cyclin D1. We therefore set out to determine whether atorvastatin and metformin could regulate the expression of STAT3 target genes in 3T3-L1 cells. Atorvastatin up-regulated the levels of the STAT3-dependent suppressed genes KLF5 and p53, while the STAT3-dependent induced gene cyclin D1 was down-regulated by atorvastatin. These regulatory effects of atorvastatin were enhanced by combination with metformin (Supplementary Fig. S2). To further investigate these effects, we transfected cells with wtSTAT3, caSTAT3, or siSTAT3. Consistent with abovementioned results, mRNA levels of KLF5 and p53 were decreased by wtSTAT3 and caSTAT3, and subsequently significantly increased following combination treatment with atorvastatin and metformin. Conversely, the levels of cyclin D1 were markedly increased by STAT3 overexpression by either wtSTAT3 or caSTAT3, and these levels were effectively decreased following combination treatment with atorvastatin and metformin (Fig. 3C). These data clearly demonstrate that atorvastatin exerts its anti-adipogenic effects at least in part by suppressing STAT3 signaling cascades, and that combination therapy with metformin results in a synergistic increase in these inhibitory activities.

### 3.6. TGF-β/Smad3 signaling is elevated by combination of atorvastatin and metformin

Transforming growth factor-β (TGF-β)/Smad3 signaling is upstream of STAT3 signaling in adipogenesis [27–29], indicating that regulation of this signaling component is also important for adipocyte differentiation. We investigated the effects of atorvastatin, alone and in combination with metformin, on TGF-β/Smad3 signaling to gain further insight into the mechanism of anti-adipogenesis. IL-6 stimulation did not affect Smad3 activation. The activity of Smad3 was inhibited by atorvastatin and metformin, but no synergistic effect was observed when the two reagents were combined. However, although TGF-β-induced Smad3 activation was not synergized in the presence of IL-6, and monotherapy with either atorvastatin or metformin did not affect this activation, activation was increased by combination treatment with atorvastatin and metformin. IL-6-induced STAT3 activation was increased in the presence of TGF-β, but was not influenced by TGF-β alone. Furthermore, this activation was decreased by atorvastatin, and this effect was synergized by metformin (Fig. 4A), indicating a mechanism of action upstream of STAT3 signaling.

To further explore these effects, we determined the expression of Smad7, an inhibitory protein of TGF-β/Smad3 signaling, and STAT3 target genes KLF5 and cyclin D1. We found that TGF-β/Smad3-associated gene expression was increased at an earlier time-point than STAT3-dependent gene expression. The expression levels of Smad7 were altered by stimulation with TGF-β, but not IL-6, and these levels were regulated by atorvastatin and metformin in a similar pattern to the effect on Smad3 activation. Furthermore, the levels of KLF5 and cyclin D1 were affected by stimulation with IL-6, but not TGF-β, and these levels were also regulated in a

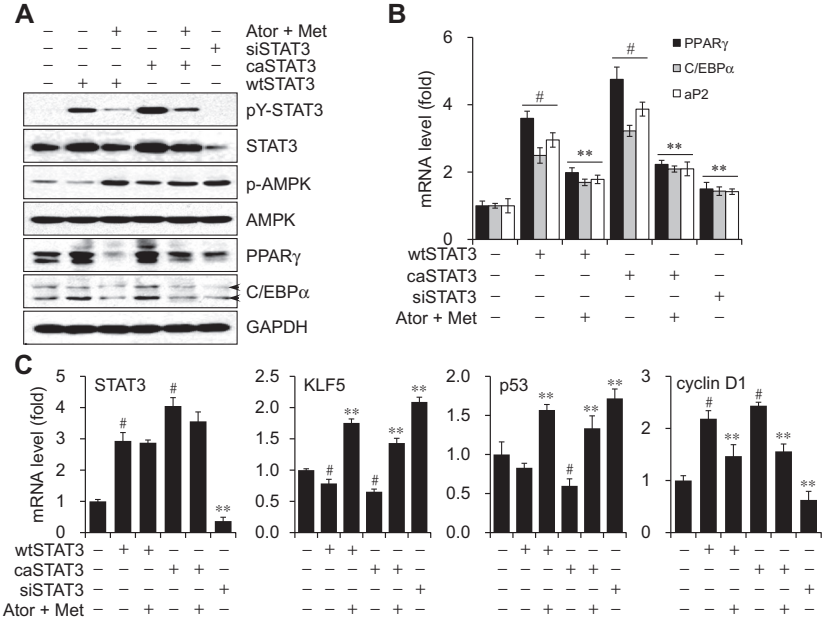


**Fig. 3.** Targeting STAT3 signaling exerts anti-adipogenic effects. 3T3-L1 cells were transfected with wtSTAT3, caSTAT3, or siSTAT3. The cells were incubated for 72 h, treated with atorvastatin (20 μM) and metformin (100 mM), and further incubated for 24 h. The levels of pY-STAT3, STAT3, AMPK activation, adipogenic and STAT3 target genes were measured by Western blot analysis (A) and quantitative real-time PCR (B and C). Results are expressed as the means ± SD of three independent experiments. $^{\#}p < 0.05$ versus mock-transfected group; $^{**}p < 0.005$ versus wtSTAT3- or caSTAT3-transfected group.

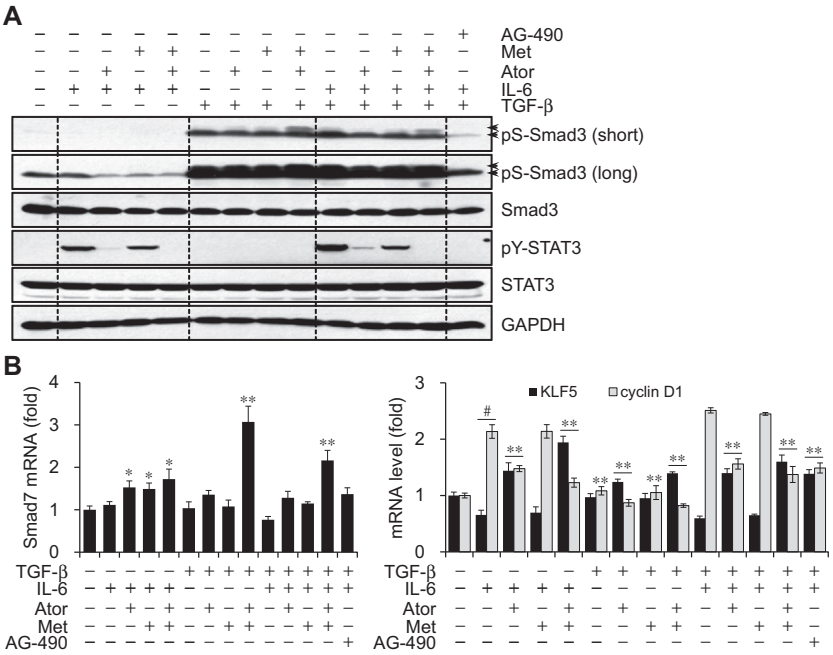


**Fig. 4.** Atorvastatin-induced elevation of TGF-β/Smad3 signaling is enhanced when combined with metformin. (A) 3T3-L1 cells were treated with atorvastatin (20 μM), metformin (100 mM), or atorvastatin (20 μM) and metformin (100 mM) for 1 h. The cells were further incubated for 15 min with IL-6 (10 ng/mL), 1 h with TGF-β (5 ng/mL), or 1 h with both IL-6 and TGF-β. Western blot analysis was performed, with GAPDH serving as a loading control. pS-Smad3 images show short and long exposure times. (B and C) Cells were incubated for 2 h (B) or 6 h (C) under the same conditions referred to in A. The levels of TGF-β/Smad3 inhibitory protein (B) and STAT3 target genes (C) were measured by quantitative real-time PCR. Results are expressed as the means ± SD of three independent experiments. $^{\#}p < 0.05$ versus vehicle group; $^{*}p < 0.05$ and $^{**}p < 0.005$ versus IL-6- or TGF-β-induced group. AG-490 (50 μM) was used as a pan-JAK/STAT3 inhibitor.

manner similar to STAT3 activation in the presence or absence of atorvastatin and/or metformin (Fig. 4B). These data indicate that TGF-β/Smad3 signaling is upstream of STAT3 signaling in adipogenesis of 3T3-L1 cells, and that inhibition of adipogenesis is elevated when atorvastatin and metformin are combined.

## 4. Discussion

There are several benefits of identifying novel pharmacological activities of currently prescribed drugs, compared to developing novel agents. The cost differential of these two processes is considerable. In addition, use of approved drugs can help to minimize side effects and reduce the need for clinical trials, because many of the properties of the compound, such as physical properties and pharmacodynamics, have been characterized. Atorvastatin and metformin are currently prescribed for dyslipidemia and type 2 diabetes, respectively. In this study, we evaluated the anti-adipogenic effects of atorvastatin alone and in combination with metformin, and elucidated the associated mechanism of action in 3T3-L1 cells. Our results demonstrate that atorvastatin inhibits STAT3- and TGF-β/Smad3-dependent adipocyte differentiation, and that these effects are enhanced when atorvastatin is combined with metformin.

The mouse 3T3-L1 pre-adipocyte cell line is a well-established model system for studying adipogenic differentiation. Adipogenesis is multistage processes that begins with mesenchymal stem cells and is induced by hormonal inducers, such as insulin-like growth factor-1 (IGF-I) and glucocorticoid, leading to sequential signaling cascades via activation of the adipogenic transcriptional regulators C/EBP family, C/EBPα, β, and δ and PPARγ [4,30]. C/EBPα and PPARγ are the key master adipogenic regulators that enhance the expression of genes associated with adipogenic differentiation [4,30]. Our results indicate that atorvastatin suppresses the mRNA and protein levels of C/EBPα and PPARγ in 3T3-L1 cells. Furthermore, atorvastatin suppresses the expression of adipogenic genes, such as aP2, during adipogenesis. In addition, atorvastatin and metformin increased AMPK activation. AMPK is a key regulator protein in the control of energy homeostasis via regulation of lipid, cholesterol and glucose metabolism [31]. Up-regulation of AMPK activation by atorvastatin and/or metformin is suggestive of an increase in energy metabolism and ATP production. Furthermore, both the inhibitory effect of atorvastatin on the expression of C/EBPα, PPARγ, and Ap2, and the atorvastatin-induced up-regulation of AMPK activation, were elevated when the drug was administered in combination with metformin. This indicates that combination therapy consisting of metformin and atorvastatin leads to increased ATP production, as a result of lipid metabolism, and inhibition of adipocyte differentiation.

Adipogenesis is a sequentially and temporally regulated process involving a variety of signaling cascades and transcription factors [32]. The STAT3 and TGF-β/Smad3 signaling pathways play critical roles in the regulation of cell growth, differentiation, and development in a wide range of biological systems, including adipogenic differentiation [5,6,27–30]. TGF-β/Smad3 signaling is upstream of STAT3 during adipogenesis [29], indicating that targeting of TGF-β/Smad3 and/or STAT3 signaling is essential for inhibition of adipocyte differentiation. In addition, both signaling cascades are associated with numerous human diseases, such as cancer, inflammation, and atherosclerosis [33,34]. Modulation of these signaling pathways is therefore of potential importance for the prevention or treatment of many diseases. Our data demonstrate that atorvastatin suppresses the differentiation of pre-adipocytes into adipocytes, and that this pharmacological effect is enhanced when atorvastatin is administered in combination with metformin. Theses inhibitory effects are dependent on the inhibition of the STAT3 and/or TGF-β/Smad3 signaling cascades, resulting in modulation of the expression of target genes, including KLF5, p53, cyclin D1, and Smad7. In addition, atorvastatin and/or

metformin increases the activation of AMPK, a key regulator of energy homeostasis, and C/EBPα and PPARγ, master transcription factors for adipogenesis.

Taken together, the results of this study demonstrate that atorvastatin suppresses adipocyte differentiation by regulating AMPK activation and C/EBPα and PPARγ expression. These effects involve the regulation of the STAT3 and TGF-β/SMAD3 signaling cascades, leading to modulation of target gene expression. These pharmacological activities of atorvastatin are synergized by combination with metformin. Atorvastatin and metformin are in current clinical use for treatment of dyslipidemia and type 2 diabetes. Our results suggest that combination therapy consisting of atorvastatin and metformin may result in decreased adipocyte differentiation, a key factor in some obesity and obesity-related diseases. Although their biological relevance requires further investigation, our findings may contribute to the development of novel treatment paradigms for obesity and obesity-related disorders.

## Conflict of interest

The authors declare no conflicts of interest.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.11.054.

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