

Original Paper

3-Acetyl-11-Keto-Beta-Boswellic Acid Inhibits Adipogenesis by Suppressing Autophagy and Inducing AMPK Phosphorylation in 3T3-L1 Cells

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Key Words

AKBA • Adipocytes • Autophagy • Adipogenesis • Molecular Docking

Abstract

Background/Aims: Adipogenesis, a process involving preadipocyte differentiation and lipid droplet accumulation, is linked to obesity. 3-Acetyl-11-keto-beta-boswellic acid (AKBA), a frankincense-derived triterpene, has anti-inflammatory and cancer properties, but its role in regulating adipocyte differentiation and adipogenesis remains unclear. **Methods:** 3T3-L1 preadipocytes were induced to differentiate into adipocytes using a differentiation cocktail, with or without varying concentrations of AKBA. Cell viability was assessed using the MTT assay, while lipid accumulation was quantified through Oil Red O staining in the differentiated 3T3-L1 adipocytes. Apoptosis was determined using the FITC (fluorescein isothiocyanate) annexin V apoptosis detection kit. Expression of pro- and anti-apoptotic proteins Bax and Bcl2, adipogenic transcription factors, lipid accumulation-related proteins, and autophagy marker proteins were analyzed via Western blotting. Additionally, molecular docking simulations were conducted to investigate the binding affinity and interactions between the bioactive compounds and the targeted proteins. **Results:** AKBA treatment inhibited adipocyte differentiation by suppressing the protein levels of essential adipogenic markers CCAAT/enhancer binding protein beta (C/EBP β), CCAAT/enhancer binding protein alpha (C/EBP α), and peroxisome proliferator-activated receptor gamma (PPAR γ). Further, AKBA treatment significantly reduced lipid accumulation and increased apoptosis in 3T3-L1 cells by increasing

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Bax/Bcl2 ratio. Notably, AKBA treatment profoundly reduced autophagy indicators, including ATG5 and LC3b protein expression in differentiated 3T3-L1 cells, concurrent with increased AMPK phosphorylation (P-AMPK). Further, a molecular docking study revealed that AKBA can block PPAR γ and ATG5. **Conclusions:** The results indicate that AKBA exhibits anti-adipogenesis effects by suppressing adipocyte-specific transcription factors C/EBP α and PPAR γ , autophagy-related marker ATG5, promoting apoptosis and activating AMPK.

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Introduction

Obesity is a global health concern [1] marked by excessive or abnormal fat mass expansion and cellular lipid buildup resulting from an imbalance in energy intake and expenditure [2]. Obesity's growing prevalence is especially concerning because it is a significant contributor to noncommunicable diseases like cancer, cardiovascular disease, and type 2 diabetes [3, 4]. Substantial evidence suggests that increased adipocyte number (hyperplasia) and size (hypertrophy) are responsible for adipose tissue expansion in the obese condition [2]. Hypertrophy is closely associated with adipogenesis, a process by which preadipocytes mature into adipocytes and accumulate lipids, to satisfy the increased lipid storage demand in obesity [2]. Consequently, adipogenesis is critical for regulating total fat mass [5].

Adipogenesis encompasses two phases: commitment and terminal differentiation. In the commitment phase, MSCs are converted into preadipocytes, which bear a morphological resemblance to their precursors but forfeit their capacity to differentiate into alternate cell lineages [6]. Preadipocytes undergo late differentiation to become adipocytes, which can produce lipids, are insulin-responsive, and manufacture proteins specific to adipocytes [6].

Adipogenesis is an essential process for fat cell formation. It involves various gene regulators, such as CCAAT/enhancer-binding proteins (C/EBPs) and peroxisome proliferator-activated receptor gamma (PPAR γ) [7, 8]. These transcription factors can activate adipocyte-specific genes associated with lipogenesis and adipogenesis, such as lipoprotein lipase, adiponectin, fatty acid synthase, and perilipin [9]. Reducing these transcription factors' expression and activity has been suggested as an effective strategy for obesity treatment. Thus, comprehending the biological processes of adipose tissue growth and the essential elements involved in its regulation is critical for future obesity prevention and treatment therapies.

The therapeutic value of bioactive compounds for disease therapy has garnered significant interest in recent years. To date, many bioactive compounds have been isolated from natural sources and tested for their efficacy in different disease models [10]. Boswellic acids, a type of pentacyclic triterpene found in plants like *Boswellia*, are commonly used to treat ailments like arthritis, psoriasis, asthma, and cancers [11-13]. Previous research has indicated that boswellic acids are well tolerated and confer beneficial effects in humans [14]. For the past ten years, we have created a library of naturally isolated and synthetically modified natural products, including a collection of medicinally relevant triterpenes extracted from frankincense [15]. Of these bioactive substances, 3-Acetyl-11-keto- β -boswellic acid (AKBA) is the most potent against cancer [13, 15, 16]. Boswellic acids (BA) and AKBA lessened STZ-mediated production of cytokines that trigger inflammation in the circulation and CD3 cell recruitment to the pancreas. Additionally, AKBA was more effective in lowering STZ-mediated hyperglycemia [17, 18]. *Boswellia serrata* (predominantly containing Boswellic acids) was also found effective in preventing diet-induced obesity, hyperlipidemia, and insulin resistance. It was further demonstrated that *Boswellia serrata* inhibited food intake and pro-inflammatory cytokine production, along with increasing anti-inflammatory adiponectin production [19]. *Boswellia serrata* gum resin drastically lowered total, LDL, and triglyceride levels in diabetic patients' blood while raising HDL cholesterol [20]. Additionally, a combination of metformin and gum resin of *Boswellia Serrata* substantially reduced blood glucose, Hemoglobin A1c (HbA1C), insulin, LDL cholesterol, and triglycerides in diabetic patients compared to metformin treatment alone [21]. Despite these promising studies on

the efficacy of Boswellic acids in obesity and diabetes, it is unclear whether it has any effect on adipocyte differentiation and lipid accumulation. More specifically, it examines the effects associated with adipogenesis and adipose tissue expansion, along with their underlying molecular and cellular mechanisms.

In the present investigation, we examined how AKBA affected variables linked to adipocyte development (C/EBP β , C/EBP α , and PPAR γ) and autophagy markers (LC3b and ATG5) in 3T3-L1 cells. Furthermore, AKBA's role in lipid accumulation and AMPK phosphorylation was examined in adipocytes. Additionally, the potential of AKBA to block adipogenesis was validated through molecular docking of pharmacological targets.

Materials and Methods

Chemicals and reagents

Oil Red O staining solution, Dulbecco's Modified Eagle Medium (DMEM), MTT assay kit, Chemiluminescence substrate, Isobutyl methylxanthine (IBMX), insulin, and dexamethasone were purchased from Sigma Aldrich (St. Louis, MO, USA). PPAR γ (Cat. #2430S), C/EBP α (Cat. #2295S), C/EBP β (Cat. #3082S), ATG5 (Cat. #2630S), P-AMPK (Thr172, Cat. #2531S), AMPK (Cat. #2532S), Bax (Cat. #2772S), Bcl2 (Cat. #2876S), and β -actin (Cat. #4967S) were obtained from Cell Signaling Technology, Danvers, MA, USA. FAS (Cat. #A0462) was purchased from Abclonal Technology (Woburn, MA, USA). LC3b (Cat. #AF4650) were obtained from Affinity Biosciences, Cincinnati, OH, USA. 3T3-L1 (Cat. #CL-173) preadipocytes were obtained from ATCC (Rockville, MD, USA) and maintained in DMEM supplemented with 10% bovine serum and 100 U/ml penicillin and streptomycin.

Extraction and purification of AKBA

The oleo-gum resin of *Boswellia sacra* was gathered from multiple sites in the Dhofar governorate of Oman (2012) and certified by the herbarium at the Natural and Medicinal Sciences Research Center, University of Nizwa, Oman (voucher specimen, BSHR-01/2020). After authentication of the sample, a 500 g sample of air-dried and crushed resin was extracted with distilled methanol (MeOH, 1.2 L) for 2-3 days with continuous and vigorous shaking after 30 min to get a yellowish residue (280 g). The MeOH extract (120 g) was analyzed using column chromatography (CC, SiO₂) using a gradient polarity of n-hexane/ethyl acetate (EtOAc) with up to 100% EtOAc, yielding twelve fractions (BS1-12) as described before [22, 23]. Fractions 5-7 were combined after TLC confirmed the presence of AKBA and then loaded onto CC (600 g; 70-230 mesh) to isolate impure AKBA (800 mg with a small amount of KBA impurity) using 30-40% EtOAc/n-hexane. After fermenting with 0.6% ethanol, the resulting sample was transferred directly to a recycling chloroform HPLC. Purified AKBA (350 mg) was eluted at a flow rate of 4 ml/min after a retention time of 42 minutes [22, 23].

Differentiation of preadipocytes into mature adipocytes

3T3-L1 preadipocytes were grown in DMEM with 10% FBS to initiate differentiation. After reaching confluence two days following plating (day 0), the cells were treated with differentiation stimulation medium containing 0.5mM isobutylmethylxanthine (IBMX), 167 nM insulin, and 1mM dexamethasone for two days (day 2). The cells were then switched to a medium that contains DMEM with 167 nM insulin for another two days (till day 4). Next, the cells were grown in DMEM medium with 10% FBS for four more days (till day 8), as shown in Fig. 1. The cells were subjected to specified treatments throughout the entire culture period (from day zero to day 8).

Cell viability assay

Cell viability was examined using the MTT test in 3T3-L1 cells treated with AKBA. The cells were plated in a 96-well plate

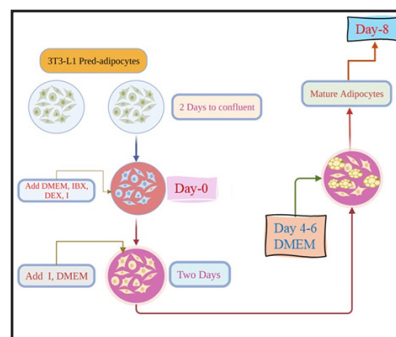


Fig. 1. Procedure for inducing differentiation of 3T3-L1 preadipocytes into mature adipocytes. DMEM; Dulbecco's modified Eagle medium, IBMX; isobutyl-methyl-xanthine, I; insulin, DEX; dexamethasone.

and cultured for 24 hours. Following treatment with varied concentrations of AKBA for 24 hours, the media was removed, and cell viability was evaluated at 570 nm with a microplate reader (Bio-Rad, USA). The results displayed a percentage change in cell viability compared to untreated control cells, determined via the following equation.

$$\% \text{ Viability} = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100 \quad (\text{Eq} - 1)$$

Oil red O staining

3T3-L1 preadipocytes were treated with 3-isobutyl-1-methylxanthine, dexamethasone, and insulin (MDI) for 8 days with and without AKBA (C-D). Undifferentiated 3T3-L1 cells without AKBA treatment were used as (C-N). The influence of AKBA on adipocyte intracellular lipid accumulation was measured by oil red O (ORO) staining. Adipocytes were fixed in 10% formalin for 1 hour, followed by permeabilization in 60% isopropanol for 5 minutes. Fixed cells were stained with Oil Red O solution in 20% isopropanol for 1 hour, then washed four times with H₂O. The lipid droplets were subsequently detected and photographed using an Evos XL Core (Invitrogen). To estimate the amount of neutral lipids, 100% isopropanol was used to extract Oil Red O from the cells, and the optical density absorbance at 510 nm was measured with a microplate reader (Bio-Rad, USA).

Preparation of cytosolic and nuclear fractions

The cytosolic fractions were prepared as previously reported [24]. Protein concentration was measured using the Bradford assay (Bio-Rad), with bovine serum albumin (BSA) as the standard, according to the manufacturer's instruction.

Western blotting

Using a mix of a proteinase inhibitor cocktail (Roche) and RIPA buffer containing 50 mM Tris, pH 7.0, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, and 1% NP-40, cells were lysed. 40 µg of proteins were separated using 12% SDS-PAGE gels and transferred to nitrocellulose membranes. Transferring gels to nitrocellulose membranes was achieved by electroblotting. The membranes were blocked with 5% nonfat milk in PBS-Tween buffer for 1 hour and then incubated overnight at 4°C with primary antibodies (C/EBPα, C/EBPβ, PPARγ, ATG5, LC3B, FAS, Cell Signaling Technology, Danvers, Massachusetts, USA) at a 1:500 dilutions. The secondary antibody (β-actin) was then incubated at 1:5000 dilutions at room temperature for 1 hour. The ECL method (Thermo Scientific, USA) was used to detect protein signals, which were then visualized using the ChemiDoc System (Bio-Rad, USA). Protein expression was evaluated densitometrically using Image J software and normalized to β-actin standard bands and total AMPK for P-AMPK. Values are presented as percentage changes relative to the control group [25]. For Bax and Bcl2 (Cell Signaling Technology, Danvers, Massachusetts, USA) identification, 20 µg of protein was loaded per well in a 15% SDS-PAGE gel and transferred on to PVDF membrane. The membrane was blocked using Intercept® (TBS) Blocking Buffer (Cat. #927-60001, LICORbio™, NE, USA) for 1h. Later, membrane was incubated with 1:1000 primary antibody overnight at 4°C. Then, the membrane was incubated with 1:2000 secondary antibody at RT for 1h. Fluorescence was imaged using Odyssey CLx Imager (LICORbio™, NE, USA). Densitometry was performed using Image Studio™ 6.0 software, (LICORbio™, NE, USA). Bax and Bcl2 values were normalized to β-actin, and the fold change was used to represent the Bax/Bcl2 ratio.

Docking simulations

Crystal structures retrieval. The crystal structural data of the PPARγ Ligand Binding Domain in conjunction with the T0070907 inhibitor (PDB ID: 6C1I), and the inactivation of Autophagy protein 5 (ATG5) by a stapled peptide (PDB ID: 7W36), was obtained from the RCSB Protein Data Bank (PDB) server to analyze the atomic interactions of the AKBA with the protein's active pockets.

The Molecular Operating Environment (MOE) software, version 2022.02 [26], was employed to restore the lost residues in the three-dimensional (3D) structures found in PPARγ and ATG5 proteins with the Loop Modeler algorithm and the Amber14:EHT forcefield [27, 28]. To add hydrogens that were missing and assign charges at the protein's terminal ends, the Quick Prep panel in MOE 2022.02 was used [26]. Additionally, the MOE software was used to add several structural deficiencies, such as missing atom types, angle definitions, forcefield parameters, Van der Waals interactions, bond formations, and residue chirality.

Molecular docking. Molecular docking is a vital tool for in silico screening, which is increasingly significant in rational drug design. Molecular docking simulates the interaction between a protein's binding site and a potential ligand, ensuring both energetic and geometric compatibility [29, 30]. We utilized the Dock function in MOE 2022.02 to conduct docking experiments between the AKBA compound and the PPAR γ as well as ATG5 proteins [26]. Initially, we re-docked the native ligand in PPAR γ to confirm the accuracy of our docking approach before examining the AKBA compounds. The validation of our docking protocol comprised of three stages. First, the ligand's geometry was optimized by rotating bonds to achieve proper alignment. Subsequently, we used the Triangle Matcher for ligand placement, generating 100 potential conformations. Each conformation's binding energy was calculated using the London dG Scoring function (Equation 2), incorporating factors such as entropy changes, flexibility loss, hydrogen bonding, metal interactions, and desolvation energies [26].

$$G = c + E_{flex} + \sum_{h-bonds} c_{HB} f_{HB} + \sum_{m-lig} c_{MF} f_M + \sum_{atoms} \Delta D_i \quad (Eq - 2)$$

In the final stage, the best conformer was chosen from the initial 100 using the GBVI/WSA dG scoring algorithm in MOE 2022.02. The binding free energies of the top 30 conformations were further calculated using a secondary formula (Equation 3) that accounts for changes in entropy, electrostatic interactions, and van der Waals forces [26].

$$\Delta G \approx c + a \left[\frac{2}{3} (\Delta E_{coul} + \Delta E_{sol}) + \Delta E_{vdw} + \beta \Delta SA_{weighted} \right] \quad (Eq - 3)$$

After validating our methodology by superimposing the native and re-docked ligands and assessing the Root Mean Square Deviation (RMSD), we proceeded to dock the AKBA with the PPAR γ and ATG5 proteins' active sites. We examined the relationships between every docked complex that was stored in PDB format. We employed the Protein-Ligand Interaction Fingerprinting (PLIF) tool available in the MOE Database viewer to perform the analysis.

Statistical analysis

The data is shown as mean $\pm$ Standard deviation (SD), with $n = 3$. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test or using an unpaired Student's t-test. The statistical significance of each comparison was assessed based on P-values lower than 0.05.

Results

Effects of AKBA on 3T3-L1 cell viability

To evaluate the effect of AKBA on 3T3-L1 cell growth, 3T3-L1 cells were induced to differentiate into adipocytes over 8 days, after which the cytotoxicity of AKBA on the differentiated adipocytes was assessed [31]. The 3T3-L1 cells were administered with AKBA at dosages ranging from 0.78 μ M to 100 μ M, and their viability was determined using the MTT assay. AKBA at concentrations of 0.78, 1.56, and 3.1 μ M had no cytotoxic effects on mature adipocytes (Fig. 2). Treatment with 6.25 to 100 μ M AKBA dramatically reduced mature adipocyte cell viability by 15 to 60% compared to the control group ($P < 0.05$, $P < 0.01$, and $P < 0.001$, Fig. 2). For subsequent experiments, we used three AKBA concentrations: 2.5 μ M (non-toxic to mature adipocytes) as well as 5 and 10 μ M (lower than IC_{50} that was determined at 53.2 μ M in matured

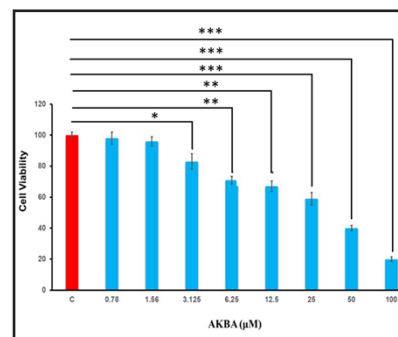


Fig. 2. AKBA reduced the viability of 3T3-L1 cells. The data is reported as mean $\pm$ standard deviation (SD) and presented as a percentage change relative to the control group. $n=3$. C; control. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. Control. P values < 0.05 were considered statistically significant for all comparisons.

adipocytes).

AKBA suppressed lipid accumulation in 3T3-L1 cells

Adipocytes retain surplus lipids or glucose as triglycerides and are essential to control lipid metabolism and energy homeostasis by providing free fatty acids and glycerol when energy is required [32]. Thus, the development of obesity is intimately linked to preadipocyte differentiation and increased lipid accumulation [33]. To determine the impact of AKBA on adipogenesis, the cytoplasmic lipid level was evaluated in mature adipocytes. 3T3-L1 preadipocytes were administered with AKBA at various concentrations (2.5, 5, 10 μM) during the differentiation phase for 5 days (days 4–8). The intracellular lipid buildup was assessed by Oil Red O (ORO) staining (Fig. 3A and 3B). Compared to undifferentiated preadipocytes (C-N), mature adipocytes (C-D) displayed a considerable increase in lipid accumulation (Fig. 3A and 3B). Microscopy revealed that 2.5 μM , 5 μM , and 10 μM AKBA significantly reduced ORO staining in differentiated 3T3-L1 adipocytes (Fig. 3A). Stained cells were eluted with isopropanol, and the cellular lipid was quantified spectrophotometrically at 510 nm using a microplate reader. We found that 2.5 μM , 5 μM , and 10 μM AKBA significantly reduced lipid buildup by 18%, 36%, and 60%, respectively, in comparison to the control group (C-D) ($P < 0.01$, $P < 0.001$, and $P < 0.001$, respectively, Fig. 3B). The data suggest that AKBA decreased lipid formation in mature adipocytes.

AKBA promoted apoptosis in 3T3-L1 cells

The present study revealed that AKBA mediated a decrease in cell viability and lipid accumulation in mature adipocytes. Next, we assessed the impact of AKBA on apoptosis in mature adipocytes. We used Annexin V-FITC and PI double staining to examine the effect of AKBA on apoptosis in mature adipocytes. During apoptosis, the membrane phospholipid phosphatidylserine translocates from the inner to the outer membrane, where it is detected by Annexin V-FITC, a phospholipid-binding protein [34]. Flow cytometry was employed to quantify early and late apoptotic cells. The results demonstrated that treatment with AKBA at various concentrations (2.5, 5.0, and 10.0 μM) for 24 hours significantly increased the number of apoptotic cells in mature 3T3-L1 adipocytes compared to the control group ($P < 0.01$, Fig. 4B). To further investigate the molecular event by which AKBA affects apoptosis, we evaluated the expression of pro- and anti-apoptotic proteins Bax and Bcl-2 which are critical regulators of the mitochondrial apoptotic pathway [35] by western blotting (Fig. 4C). AKBA treatment tended to increase Bax expression, while 10 μM AKBA markedly reduced Bcl-2 levels ($P < 0.01$, Fig. 4D) resulting in an elevated Bax/Bcl2 ratio ($P < 0.05$, Fig.

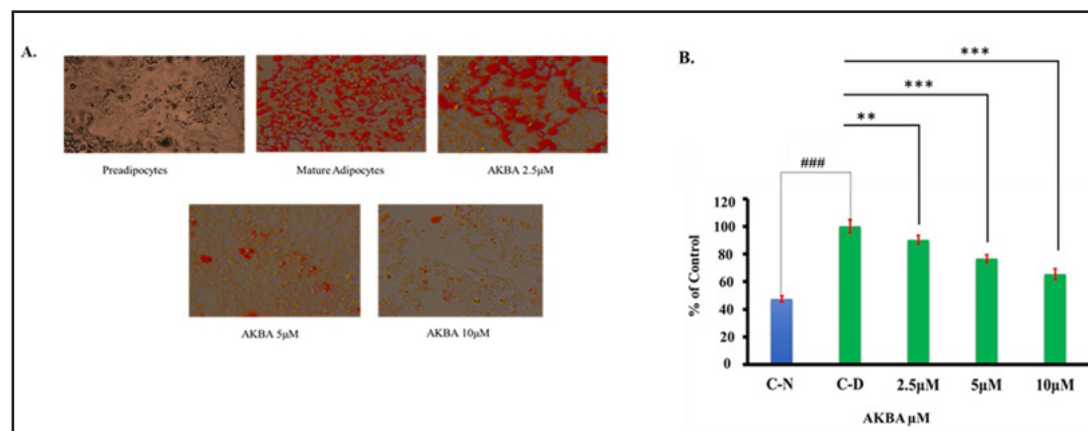


Fig. 3. AKBA suppressed lipid buildup in differentiated 3T3-L1 cells. (A) Representative images (40x magnification) of Oil Red O staining are shown. (B) Lipid quantification. Data are reported as mean $\pm$ SD, $n=3$. Statistical analysis was performed using an unpaired Student's t-test; ### $P < 0.001$ vs. C-N and one-way ANOVA with Tukey's post hoc test; ** $P < 0.01$, *** $P < 0.001$ vs. control group (C-D). P values < 0.05 were considered statistically significant for all comparisons.

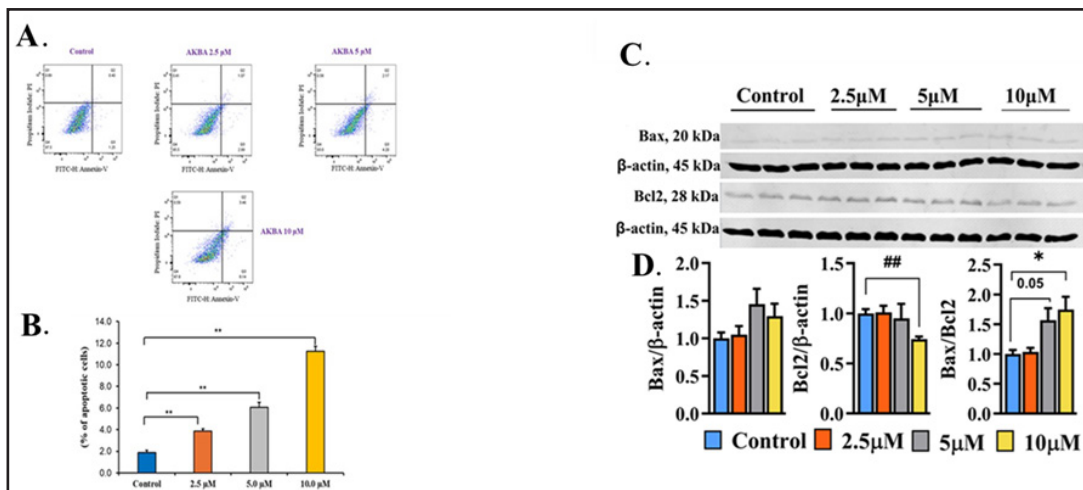


Fig. 4. AKBA treatment promoted apoptosis in 3T3-L1 mature adipocytes. (A) Annexin-V-FITC/PI staining in 3T3-L1 cells after treatment with AKBA. (B) The percentage of apoptotic cells in 3T3-L1 cells after treatment with AKBA. Q1: (AnnexinV-FITC)⁻/PI⁺, necrotic cells or a few late apoptotic cells, mechanically damaged cells; Q2: (AnnexinV-FITC)⁺/PI⁺, late apoptotic cells; Q3: (AnnexinV-FITC)⁺/PI⁻, early late apoptotic cells; Q4: (AnnexinV-FITC)⁻/PI⁻, live cells. Data are reported as mean ± SD, n=3. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test. **P<0.01 vs. control group. (C) Immunoblots for Bax and Bcl2 with β-actin as loading control. (D) The bar graph represents protein band density. Data are represented as Mean ± SEM, n=3, and reported as fold change compared to the control. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test; * P < 0.05, and unpaired Student's t-test; ## P < 0.01. P values < 0.05 were considered statistically significant for all comparisons.

4D) and enhanced apoptosis. The increased apoptosis following AKBA treatment may account for the observed decrease in cell viability and lipid accumulation in this study.

AKBA reduced adipogenic and lipogenic factors in 3T3-L1 cells

PPAR γ and C/EBP α are essentially involved in adipocyte differentiation [8]. They can induce adipose-specific genes, including fatty acid synthase (FAS), which is responsible for lipid synthesis [9]. We evaluated the expression levels of C/EBP β , PPAR γ , and C/EBP α , key transcription factors implicated in adipocyte differentiation, to understand the mechanism of AKBA-mediated reduction in intracellular lipid content. 2.5 μ M, 5 μ M, and 10 μ M of AKBA treatment significantly reduced the protein levels of C/EBP β (10%-55%), PPAR γ (20% to 65%), and C/EBP α (20%-70%) and lipogenic factor FAS (18 to 55%) compared to control treatment (Fig. 5A and 5B). These findings suggest that AKBA-mediated reduction of adipogenic and lipogenic transcription factors may be accountable in part for the lowered lipid content detected after AKBA administration.

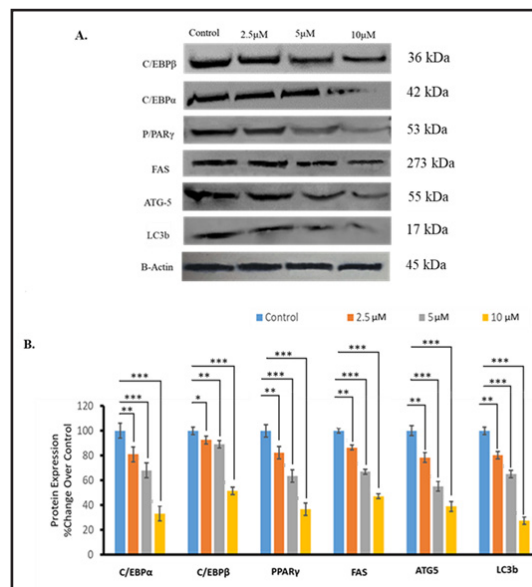


Fig. 5. AKBA treatment affected expression of adipocyte differentiation, autophagy, and lipogenic markers. (A) Immunoblots for proteins related to adipocyte differentiation, autophagy, and lipid accumulation. Representative blots are shown. (B) The bar graph represents protein band density. Data are mean ± SD and reported as percentage change compared to the control. n=3. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test. *P<0.5, **P<0.01, ***P<0.001 vs. control. P values < 0.05 were considered statistically significant for all comparisons.

AKBA suppressed protein expression linked to autophagy in 3T3-L1 adipocytes

Autophagy has a substantial influence on adipocyte development [36]. Thus, we checked the expression of autophagy-related proteins following AKBA treatment. AKBA treatment with 2.5 μ M, 5 μ M, and 10 μ M markedly reduced ATG5 protein expression by 20%, 40%, and 50%, respectively, compared to the control treatment ($P < 0.01$, $P < 0.001$, $P < 0.001$, respectively, Fig. 5A and 5B). The same AKBA treatment reduced LC3b protein expression by 20%, 35%, and 65%, respectively than the control treatment ($P < 0.01$, $P < 0.001$, $P < 0.001$, respectively, Fig. 5A and 5B). These results indicate that AKBA has the potential to suppress autophagy in adipocytes.

AKBA increased AMPK phosphorylation in 3T3-L1 cells

AMP-activated protein kinase (AMPK) is crucial for adipocyte proliferation, differentiation, and lipid metabolism [37]. Additionally, increased AMPK phosphorylation, an indicator of AMPK activation, has demonstrated anti-adipogenic effects [37]. Next, we evaluated whether AKBA-mediated repression of adipogenic transcription factor expression and lipid accumulation in mature adipocytes involves AMPK activation. Western Blot results indicated that the phosphorylation of AMPK was significantly enhanced 30%, 60%, and 100% upon treatment with 2.5 μ M, 5 μ M, and 10 μ M of AKBA ($P < 0.01$, $P < 0.01$, and $P < 0.001$, respectively, Fig. 6A and 6B). The findings suggest that AKBA promotes AMPK phosphorylation (Thr172), which may contribute to blocking adipocyte differentiation and reducing lipid accumulation in 3T3-L1 cells.

Molecular docking results

The molecular docking was performed to explore the atomic interactions between the PPAR γ (PDB ID: 6C1I) and ATG5 (PDB ID: 7W36) proteins retrieved from the protein databank server (Fig. 7A). The molecular interactions results reveal critical interactions between the reference ligand 6C1I and PPAR γ (T0070907), with noteworthy bonds at SER289 and HIS323, both involving hydrogen bonds that stabilize the ligand within the binding site (Fig. 7B). For the ATG5 peptide inhibitor 7W36, significant ionic bonds are observed with Asp3 from both Lys3 and Arg4 and a powerful bond with Asp10 from Arg11, indicating key sites for drug targeting (Fig. 7C). Additionally, hydrogen bond interactions involving Arg13, Gln17, His5, and Trp2 of the stapled peptide with ATG5 active site residues suggest a strong binding profile that could be pivotal for inhibitory efficacy (Fig. 7D). The co-crystallized ligand of the PPAR γ was redocked in the active pocket of the protein to validate the docking protocol and shows RMSD value of 0.684 Å with a docking score of -5.67 kcal/mol, which shows a favorable RMSD and accuracy of the docking protocol (Fig. 8A). The AKBA, our compound of interest, demonstrates potent interactions with PPAR γ , achieving a docking score of

Fig. 6. Effects of AKBA on AMPK phosphorylation in 3T3-L1 cells. (A) Immunoblot for P-AMPK. Representative blots are shown. (B) The bar graph represents protein band density. Data are mean $\pm$ SD and presented as percentage change over control following normalization to AMPK. $n = 3$. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test. ** $P < 0.01$, *** $P < 0.001$ vs. control group. P values < 0.05 were considered statistically significant for all comparisons.

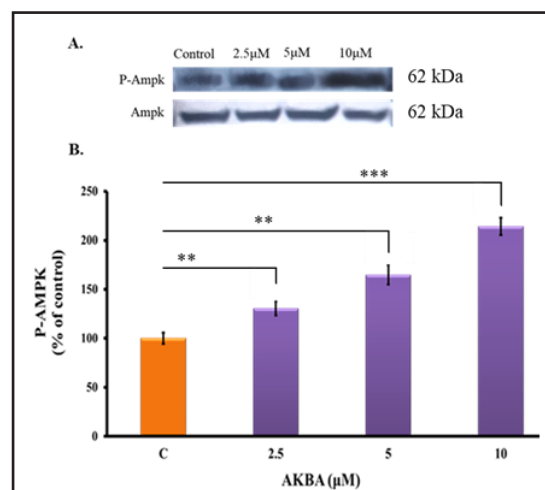


Fig. 7. Detailed visual representation of the (A) PPAR γ protein having (B) T0070907 ligand attached in the active site and (C) ATG5 structures having (D) stapled peptide in the active site retrieved from the protein data bank server with PDB IDs 6C1I and 7W36.

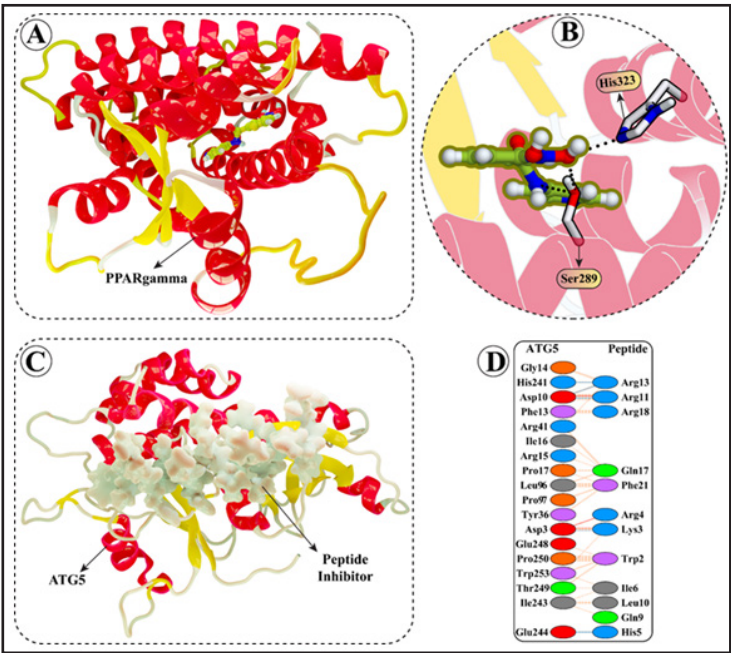
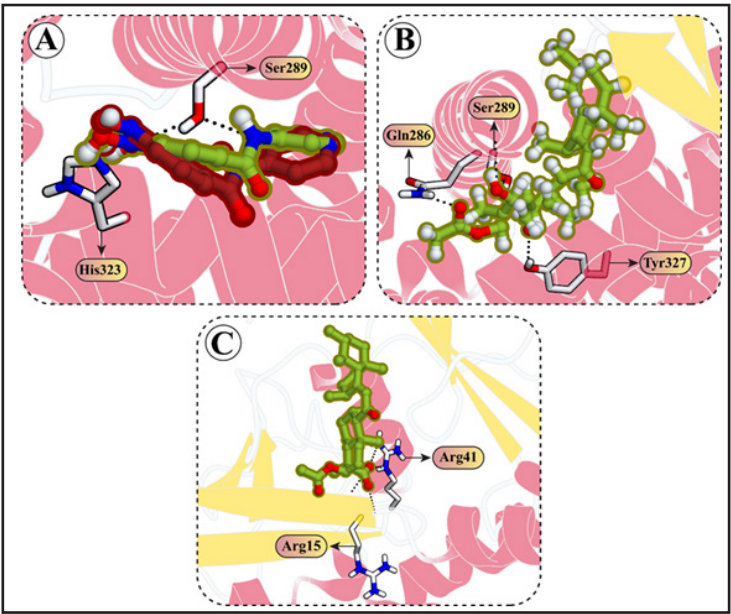


Fig. 8. Docking interactions of AKBA with PPAR γ and ATG5 proteins. (A) The co-crystallized ligand of PPAR γ , having PDB ID 6C1I, was redocked, resulting in an RMSD value of 0.684 Å between the co-crystallized (shown in green) and redocked (shown in red) structures. (B) 3D visualization of the interaction between AKBA and the active site residues of PPAR γ . (C) Molecular interactions of AKBA with the active site residues of the ATG5 protein.



-7.67 kcal/mol, and with ATG5, where it attains a docking score of -6.57 kcal/mol. AKBA demonstrates potent interactions with both PPAR γ and ATG5 active site residues. In the PPAR γ active site, AKBA forms hydrogen bonds with TYR327 and GLN286, positioning it effectively within the binding domain (Fig. 8B). With ATG5, AKBA shows strong and repeated interactions with ARG41, noticeable both hydrogen- and ionic bonds, suggesting a highly favorable binding affinity and specificity (Fig. 8C). These interactions, particularly those with high binding energies, are crucial for understanding

Table 1. Summary of molecular docking interactions of the reference ligand 6C1I with PPAR γ , the ATG5 peptide inhibitor with active site residues, and the interactions of AKBA with the active site residues of both PPAR γ and ATG5

Compounds	Ligand Atoms	Receptor Atoms	Residues	Bond Type	Distance (Å)	Energy (kcal/mol)
6C1I	N12	OG	SER289	H-donor	2.96	-2.9
	O09	NE2	HIS323	H-donor	2.99	-2.7
	Lys3	OD2	Asp3	Ionic	2.14	-13.79
	Arg4	O	Asp3	Ionic	3.69	-2.04
	Arg11	O	Asp10	Ionic	3.11	-20.1
7W36	Arg13	OD2	Asp10	H-acceptor	2.89	-6.2
	Gln17	O	Arg41	H-acceptor	3.53	-0.6
	Arg13	O	His241	H-acceptor	3.06	-4.9
	His5	O	Glu244	H-acceptor	3.13	-1.7
	Trp2	O	Met246	H-acceptor	3.99	-1
AKBA-PPAR γ	Trp2	O	Trp253	PI-Hydrogen	4.02	-0.5
	O36	OH	TYR327	H-acceptor	2.59	-0.8
	O37	CA	GLN286	H-acceptor	3.02	-0.5
	O37	OE	SER289	H-acceptor	2.95	-0.6
	O36	NE	ARG41	H-acceptor	2.86	-6.5
AKBA-ATG5	O36	NE	ARG41	H-acceptor	2.86	-6.5
	O37	N	ARG15	H-acceptor	3.51	-0.5
	O37	N	ARG15	H-acceptor	3.51	-0.5
	O36	NE	ARG41	Ionic	2.86	-5.5
	O36	NH2	ARG41	Ionic	3.39	-2.4
AKBA-ATG5	O36	NE	ARG41	Ionic	2.86	-5.5
	O36	NH2	ARG41	Ionic	3.39	-2.4

the molecular basis of AKBA's action and its potential as a targeting therapeutic agent against PPAR γ and ATG5. The detailed interactions of the reference inhibitors and AKBA with PPAR γ and ATG5 are shown in Table 1.

Discussion

The current study uncovered that AKBA blocked adipocyte development by lowering the expression of adipogenic gene regulators C/EBP α , C/EBP β , and PPAR γ . Furthermore, AKBA inhibited lipid formation in 3T3-L1 cells while downregulating lipogenic protein FAS expression.

Crucially, AKBA treatment stimulated AMPK phosphorylation and repressed autophagy-related proteins ATG5 and LC3b. Molecular docking studies showed that AKBA significantly docked with PPAR γ and ATG5. Our study is the first to demonstrate the involvement of autophagy and AMPK in controlling adipocyte differentiation.

Obesity is a public health issue that arises from the enlargement (hypertrophy) and increase in number (hyperplasia) of adipocytes, as well as immune cell infiltration in adipose tissue [38]. Significantly, apoptosis in both progenitor and mature adipocyte cells plays a crucial role in regulating overall body adipose mass [39]. Several natural compounds have shown promise in inducing apoptosis in 3T3-L1 adipocytes [40-42]. Mechanistically, apoptosis can be triggered through two distinct signaling pathways: the extrinsic pathway, which involves death receptors on the cell surface, and the intrinsic pathway, which operates through the mitochondria [43]. Bax and Bcl2 are key mitochondrial apoptosis regulators, with Bcl-2 functioning as an anti-apoptotic protein and Bax as a pro-apoptotic mediator. The elevated Bax/Bcl2 ratio is a critical determinant of apoptosis induction [44]. In the present study, AKBA treatment demonstrated an increased trend of Bax and decreased Bcl2 expression, elevating the Bax/Bcl2 ratio in mature 3T3-L1 adipocytes, suggesting that AKBA-induced apoptosis involves Bcl2 family proteins and the mitochondrial pathway. Several bioactive compounds were found effective in inducing apoptosis in mature adipocytes by regulating Bax and Bcl2 [42, 45]. However, the contribution of cytochrome C and caspase-3, both central regulators of apoptosis, remains undefined in AKBA-mediated apoptosis and warrants further investigation. Further, AMPK activation can trigger proapoptotic protein expression by regulating AKT [46]. We observed that AKBA increases AMPK phosphorylation and induces apoptosis in mature adipocytes, potentially contributing to reduced cell viability and inhibited lipid accumulation. However, the precise mechanisms by which AKBA triggers apoptosis in mature adipocytes remain unclear and require further investigation.

Transcription factors and signaling pathways critically regulate adipogenesis. In early differentiation, expression levels of C/EBP β / δ are high, stimulating PPAR γ and C/EBP α , essential transcription factors for adipogenesis [8]. During differentiation's terminal stage, PPAR γ and C/EBP α induce adipocyte-specific genes such as FAS and LPL [7]. FAS is crucial for fatty acid production in 3T3-L1 adipocytes [47]. AKBA treatment markedly reduced these factors at the protein level. We have demonstrated that AKBA dose-dependently reduced the expression of C/EBP β , PPAR γ , and C/EBP α and the lipogenic protein FAS, which may be responsible for AKBA-mediated inhibition of adipocyte differentiation and lipid accumulation in differentiated adipocytes. Liu et al. previously reported AKBA-mediated reduction of adipogenic transcription factors and induction of lipolysis in 3T3-L1 cells [48]. In their study, AKBA was applied to fully differentiated adipocytes, whereas in ours, 3T3-L1 preadipocytes were treated with AKBA during the differentiation process. Our findings show that AKBA inhibits adipogenesis by suppressing C/EBP β , C/EBP α , and PPAR- γ , while concurrently inducing apoptosis.

Autophagy, a critical cellular breakdown system, begins with the production of autophagosomes, which then destroy organelles and proteins [49]. Autophagy activation and inhibition are associated with a large number of pathophysiological conditions, such as cancer, diabetes, and especially neurodegenerative disorders [50]. New evidence indicates

that autophagy has an essential part in lipid buildup in fat cells [51]. Singh et al. found that inhibiting autophagy limited TG accumulation in 3T3-L1 adipocytes and suppressed the expression of adipocyte differentiation marker proteins [52]. Notably, autophagy inactivation via ATG7 deletion altered adipogenesis, leading to the development of adipose tissue with a variety of anti-obesity and anti-diabetic features. This study not only demonstrated autophagy's physiological importance in adipogenesis, but it may also open up intriguing possibilities for treating obesity and diabetes [53, 54].

A study using primary MEFs revealed that autophagy is induced during adipogenesis, and atg5 deficiency drastically lowered adipogenesis efficiency. While ATG5 deletion did not significantly affect early events, it may cause adipogenesis arrest at later stages and can lead to apoptosis. The study also demonstrated that ATG5 function is crucial for adipogenesis, suggesting autophagy's involvement in adipogenesis [53, 54]. Interestingly, one study demonstrated that autophagy genes are controlled during the development of adipocytes [55]. Notably, this regulation was mediated by adipogenic transcription factors like C/EBP β and PPAR γ . The AKBA-mediated reduction in ATG5 observed in our study could explain the lower expression of the adipocyte differentiation marker, suppression of adipocyte differentiation, and lower lipid levels in adipocytes.

AMPK is a metabolic sensor that governs cellular energy homeostasis [56]. Its activation inhibits fatty acids, cholesterol, and triglycerides synthesis and stimulates fatty acid oxidation. Additionally, AMPK inhibits ACC1, SREBP1c, and FAS, preventing the accumulation of lipids and sterols [57]. Significantly, AMPK activation can inhibit adipocyte differentiation by repressing the crucial adipogenic transcription factors, including C/EBP α and PPAR γ [58]. Our study revealed that AKBA activated AMPK phosphorylation while suppressing autophagy proteins ATG5 and LC3b and inhibited adipocyte differentiation. Important evidence suggests that AMPK activation also stimulates autophagy activity [59]. Discrepancies in findings regarding the impact of AMPK activation on autophagy and adipogenesis exist. Rahman et al. showed that *Bifidobacterium longum* subsp. *infantis* YB0411 (YB) treatment inhibited adipocyte differentiation by suppressing adipogenic transcription factors such as C/EBP α , C/EBP β , and PPAR γ . Further, YB-mediated blockade of adipocyte differentiation was concomitant with the suppression of autophagy marker p62 and LC3B and AMPK activation [60]. In contrast, Li et al. found that icariin, a flavonoid, blocked adipocyte differentiation by suppressing AMPK activation and autophagy [61].

Overall, our findings suggest that AKBA may inhibit adipogenesis through AMPK activation and autophagy regulation and via apoptosis induction.

Limitations of our study

Despite the demonstrated effectiveness of AKBA in inhibiting adipocyte differentiation and lipid accumulation, this study has several limitations. First, the precise roles of AMPK and autophagy in AKBA-mediated regulation of adipogenesis remain unclear. Although AKBA suppressed autophagy markers in adipocytes, its direct impact on adipogenesis remains unclear, partly because autophagy is a dynamic process and static measurements of ATG5 and LC3b do not adequately reflect autophagic flux. Therefore, future studies should employ lysosomal inhibitors such as bafilomycin A1 or chloroquine to more reliably assess autophagy and clarify the role of AKBA in regulating adipocyte differentiation. Additionally, comparing AKBA's binding affinities with known PPAR γ and ATG5 ligands, along with conducting molecular dynamics simulations, would strengthen the biological relevance of our docking results. These aspects should be addressed in future studies. Finally, while the present study was limited to *in vitro* experiments, *in vivo* outcomes may differ due to factors such as pharmacokinetics and tissue distribution. Therefore, future animal studies are planned to evaluate the anti-obesity effects of AKBA, with particular emphasis on its bioavailability and impact on adipose tissue.

Conclusion

AKBA inhibited adipocyte differentiation by repressing adipogenic factors such as C/EBP α , C/EBP β , and PPAR γ . Further, AKBA induced apoptosis by increasing the Bax/Bcl2 and reducing lipid accumulation and FAS protein expression in differentiated adipocytes. Importantly, AKBA induced AMPK phosphorylation and suppressed autophagy-related proteins ATG5 and LC3b. Molecular docking studies have demonstrated the proficient binding of AKBA to PPAR γ and ATG5 (Fig. 9). Altogether, the efficacy of AKBA in inhibiting adipogenesis has been demonstrated through its targeting of AMPK, PPAR γ , ATG5, and the mitochondrial apoptotic pathway. These findings underscore the potential of AKBA as a natural anti-obesity therapeutic agent. Nevertheless, further *in vivo* research is necessary to validate these *in vitro* findings.

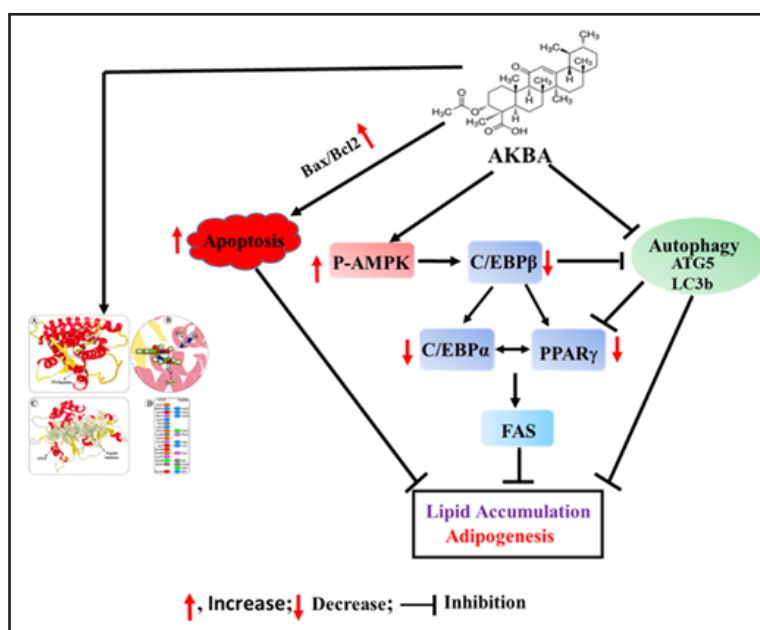


Fig. 9. An illustration of how AKBA regulates adipogenesis. AKBA treatment may inhibit adipocyte differentiation factors (C/EBP β and PPAR γ) and lipid accumulation by inducing AMPK phosphorylation and suppressing autophagy in 3T3-L1 cells. Further, AKBA may promote apoptosis by increasing the Bax/Bcl2 ratio in 3T3-L1 cells.

Abbreviations

AKBA: 3-acetyl-11-keto- β -Boswellic acid; ATG5: autophagy related 5 gene; C/EBPs: CCAAT/enhancer-binding proteins; LC3b: microtubule-associated protein 1 light chain 3b; PPAR γ : peroxisome proliferator-activated receptor gamma; AMPK: AMP-activated protein kinase; HbA1c: Hemoglobin A1c; FAS: fatty-acid-synthase; LPL: lipoprotein lipase

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Author Contributions

Faizullah Khan: Conceptualization, Data curation, Statistical analysis, Writing- Original draft preparation, read and approved the final manuscript. **Muhammad Waqas:** Methodology,

Data curation, read and approved the final manuscript. **Hassan Moghtaderi:** Methodology, read and approved the final manuscript. **Najeeb Ur Rehman:** Methodology, Writing - review & editing, read and approved the final manuscript. **Satya Kumar Avula:** Methodology, Writing - review & editing, read and approved the final manuscript. **Haroon Khan:** Writing - review & editing, read and approved the final manuscript. **Mahua Choudhury:** Writing - review & editing, read and approved the final manuscript. **Ahmed Al-Harrasi:** Writing - review & editing, read and approved the final manuscript. **Shaikh Mizanoor Rahman:** Conceptualization, Supervision, Writing- Original draft preparation, Writing-review & editing, read and approved the final manuscript. All authors read and approved the final version of the manuscript.

Data availability

Data will be made available on request.

AI Disclosure

No AI tools have been used to create this work.

Disclosure Statement

The authors declare that they have no conflicts of interest.

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