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ORIGINAL RESEARCH

Aaptamine Inhibits Lipid Accumulation and *Pparg* and *Slc2a4* Expression While Maintaining the Methylation of the *Pparg* Promoter During 3T3-L1 Adipocyte Differentiation

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Purpose: Excessive adipogenesis plays a role in the development of obesity and related metabolic disorders. Aaptamine is an alkaloid compound that has been proven to have various effects, however, no studies have yet investigated its effects on adipogenesis. This study aims to examine whether aaptamine inhibits lipid accumulation and *Pparg* and *Slc2a4*, two important genes in adipogenesis, mRNA expression, and increases the methylation of the *Pparg* promoter. This study strengthens the insights regarding these genes regulation, with future research potentially expanding to other adipogenic regulators for a broader perspective.

Methods: The effects of aaptamine (0 μM, 25 μM and 50 μM) were investigated in 3T3-L1 preadipocytes. The adipocytes were differentiated using a medium containing 3-isobutyl-1-methylxanthine, dexamethasone, and insulin. Cell viability was evaluated by the MTT assay, gene expression was analyzed by RT-qPCR, and lipid accumulation was determined using Oil Red O staining. Pyrosequencing was performed to measure the methylation of the *Pparg* promoter region.

Results: Aaptamine treatment significantly dose-dependently decreased lipid accumulation and inhibited *Pparg* and *Slc2a4* mRNA expression. However, there were no significant differences in the methylation level of the *Pparg* promoter.

Conclusion: Aaptamine inhibits lipid accumulation and *Pparg* and *Slc2a4* mRNA expression while maintaining the methylation level of the *Pparg* promoter during 3T3-L1 adipocyte differentiation.

Keywords: aaptamine, adipocyte differentiation, DNA methylation, *Pparg*, *Slc2a4*

Introduction

Obesity has become a serious public health problem in both developed and developing countries and its prevalence continues to increase year by year.[1] Basic Health Research of Indonesia reported that the prevalence of obesity in Indonesia increased from 26.6% in 2013 to 35.4% in 2018.[2,3] Obesity is the condition of excessive body weight caused by fat mass accumulation in adipocytes,[4] with surplus energy causing adipocyte hypertrophy and hyperplasia.[5,6] During hyperplasia, preadipocytes differentiate into mature adipocytes through adipogenesis regulated by *Pparg* and *Cebpa*. *Pparg* increases the expression of cyclin-dependent kinase inhibitor p21 and G0−G1, consequently promoting cell-cycle withdrawal.[7] *Pparg* and *Cebpa* also regulate the downstream genes in adipogenesis such as *Slc2a4* which encodes glucose transporter protein type 4 (GLUT4).[8,9]

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Epigenetics refers to changes in gene expression without altering the DNA sequence through various mechanisms including DNA methylation, posttranslational histone modifications, and non-coding RNA (ncRNA).[10] DNA methylation involves the addition of a methyl group to cytosine to produce 5-methylcytosine to regulate gene expression. *Pparg* gene expression is inhibited by methylation facilitated by DNMT1, DNMT3a, and DNMT3b in the promoter region.[11]

Typically, white adipose tissue is formed due to excess energy[12] and 3T3-L1, fibroblast-like preadipocytes derived from mouse embryos, representing certain white adipose tissue genes.[13,14] This cell line can be differentiated into mature adipocytes with a specific adipogenic cocktail (insulin, dexamethasone, and isobutyl methylxanthine, IBMX)[15] for in vitro research on white adipose tissue. Furthermore, 3T3-L1 cells are frequently used in studies of adipogenesis, adipocyte metabolism, and metabolism-related hormones.[16]

Several marine organisms, mainly sponges, serve as a reservoir of bioactive compounds[17] such as aaptamine, demethyloxyaaptamine, and isoaaptamine from *Aaptos suberitoides* which exert beneficial effects on adipocyte differentiation.[18,19] Several studies have shown that aaptamine possesses antimicrobial, antifungal, antifouling, cytotoxic, antiviral, antioxidant, and anticancer preventive activities. However, to date, there has been no research on the effects of aaptamine on adipocyte differentiation.[19] Inhibiting adipogenesis could offer a potential strategy to prevent obesity, even though energy balance remains the primary factor. Targeting adipogenesis may still be beneficial in preventing excessive fat accumulation, particularly in conditions of metabolic dysfunction. The clinical implications of these findings suggest that aaptamine holds significant potential as a therapeutic agent in managing disease like cancer and noncommunicable diseases. Its bioactive properties, particularly its antimicrobial, antioxidant, cytotoxic, and other important effects, could contribute to improved treatment strategies. Aaptamine has demonstrated efficacy in modulating key G protein-coupled receptors (GPCR) pathways, including chemokine receptors (immune cell migration and cancer), adrenoreceptors (cardiovascular, diabetes, and neural activity), and dopamine receptors (neural activity), highlighting its potential for novel pharmacological applications.[19] Its established safety profile and bioavailability further support its clinical relevance. Given these properties, this study aims to investigate the effects of aaptamine on 3T3-L1 adipocyte differentiation, focusing on lipid accumulation, *Pparg* and *Slc2a4* mRNA expression, and *Pparg* DNA methylation. The selection of *Pparg* and *Slc2a4* in this study was based on their crucial roles in adipogenesis. *Pparg* is a well-established master regulator of adipocyte differentiation, whereas *Slc2a4* has been recently implicated in lipid metabolism and adipogenesis but remains underexplored. Although other genes are also involved in adipogenesis, we focused on these two to provide a detailed mechanistic insight. While future studies may expand the analysis to include additional adipogenic regulators for a broader perspective, our findings offer a foundational reference for developing effective obesity prevention and treatment strategies.

Materials and Methods

Research Design and Materials

Aaptamine (cat no. SC-202899) was purchased from Santa Cruz, fetal bovine serum (FBS; cat no. 10270–106), phosphate-buffered saline (PBS; cat no. 70011004), and Dulbecco's Modified Eagle's medium (DMEM; cat no. 11995 065) from Gibco, insulin (cat no. I0516), IBMX (3-isobutyl-1-methylxanthine; cat no. I5879), dexamethasone (cat no. D4902), and DMSO (cat no. D8418) from Sigma Aldrich, and (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; cat no. M6494) from ThermoFisher. A stock solution of aaptamine was prepared by dissolving 1 mg of aaptamine in 1 mL DMSO. The 3T3-L1 preadipocytes were gift from Dr. Afiat Berbudi (Faculty of Medicine, Universitas Padjadjaran) who obtained the cells from the German Diabetes Centre, Düsseldorf, and the Institute of Pharmacology and Toxicology, University Hospital of Bonn. The Research Ethics Committee of Universitas Padjadjaran approved the utilization of the cells in this study (research protocol No. 1186/UN6.KEP/EC/2024).

MTT Assay

The potential toxic effects of aaptamine on the 3T3-L1 cell line were investigated using the MTT assay. The cells were seeded into 96-well plates and treated with aaptamine (0 μM, 5 μM, 10 μM, 25 μM, 50 μM, and 100 μM) for 72 hours. Based on previous study, these dosages are physiologically relevant for in vivo applications.[20] MTT reagent was added to

the cells and incubated for 4 hours before the reaction was stopped with the addition of DMSO. The absorbance was measured at 550 nm to determine the percent cell death.

Cell Culture and Differentiation

The 3T3-L1 cell line was grown in 10% DMEM at 37°C and 5% CO_2 until confluent. The cells were treated and differentiated according to the protocol developed by Ariyanto et al[1] as shown in Figure 1. The cells were seeded into 12-well plates and cultured in medium without aaptamine (control), medium with 25 µM aaptamine, and medium with 50 µM aaptamine before differentiation with the MDI cocktail containing 0.5 mm IBMX, 0.25 µM dexamethasone, and 1 µg/mL insulin on day 0. The medium was changed every two days until the adipocytes reached maturity and the cells were harvested for analysis on day 13.

Lipid Accumulation

The cells were washed twice with PBS before fixing with 4% formaldehyde for 10 minutes. The cells were then washed twice with PBS and 60% isopropanol for 1 minute before treatment with Oil Red O staining solution for 15 minutes. Excess Oil Red O was removed with PBS and 98% isopropanol was added for 5 mins before the absorbance was measured at 550 nm to quantify lipid accumulation.

RT-qPCR

Total RNA was isolated using the Quick RNA™ Miniprep Kit (Zymo Research Corporation) according to the manufacturer's instructions and reverse transcribed using the SensiFAST™ cDNA Synthesis Kit (Bioline Reagents Ltd). RNA concentration and purity were quantified via spectrophotometry (NanoDrop™, A260/A280 ratios = 1.8–2.0), while RNA integrity was confirmed by 1% agarose gel electrophoresis. RT-qPCR was conducted using gene-specific primers (Table 1) and the SensiFAST™ SYBR® No-ROX Kit based on the manufacturer's instructions and involved 40 cycles of denaturation at 95°C for 5 seconds and annealing at 60−65°C for 20 seconds. *Pparg* and *Slc2a4* mRNA expression was normalized to the housekeeping gene, *Gapdh*.

Pyrosequencing

Bisulfite PCR was performed following the manufacturer's instructions using 1 µL of bisulfite-treated DNA as the template. The target location for the *Pparg* gene is on chromosome 6: 115,337,600–115,339,801 (promoter region). The primers used were forward 5′-TGGTTATTTTAGGAGGTGTGT-3′ and reverse 5′-TGGTTATTTTAGGAGGTGTGT-3′. After PCR, the biotin-labeled DNA strand was captured using streptavidin-coated beads and was then incubated with the sequencing primer 5′-ATATTATTYGGTTATTTAAA AAGATAGAGGTATTTATTATAA-3′ to determine the methylation level of the designated CpG site in the promoter region.

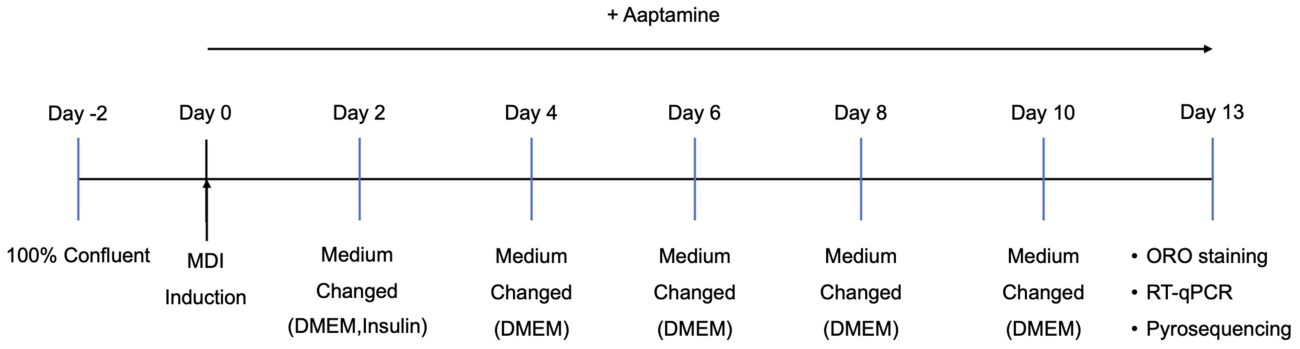

Figure 1 Adipocyte differentiation protocol.
Abbreviations: RT-qPCR, quantitative reverse transcription polymerase chain reaction; ORO, Oil red O; MDI, 0.5 mm isobutyl methylxanthine, 0.25 µM dexamethasone, and 1 µg/mL insulin-containing medium; Insulin, 1 µg/mL insulin-containing medium.

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Table 1 Primer Sequences for RT-qPCR Analysis

No.	Primer	Sequence
1	*Pparg*-Forward	5'-CAAGAATACCAAAGTGCGATCAA-3'
2	*Pparg*-Reverse	5'-GAGCTGGGTCTTTTCAGAATAATAAG-3'
3	*Slc2a4*-Forward	5'-TAACTTCATTGTCGGCATGGG-3'
4	*Slc2a4*-Reverse	5'-TGAAGAAGCCAAGCAGGAGG-3'
5	*Gapdh*-Forward	5' CAT CAG CAA TGC CTC CTG C-3'
6	*Gapdh*-Reverse	5' ATG GAC TGT GGT CAT GAG TCC-3'

Abbreviation: RT-qPCR, Quantitative reverse transcription polymerase chain reaction.

Statistical Analysis

Statistical analysis was performed in SPSS version 26 for Windows (IBM software, New York) and graphs were created using GraphPad Prism version 8.3 for Windows (GraphPad software, Inc., San Diego, CA). Normality of data distribution was verified using the Shapiro–Wilk test. Differences between treatments were examined using one-way ANOVA followed by Tukey's HSD for normally distributed data or the Kruskal Wallis test for non-normally distributed data. A p-value < 0.05 was considered statistically significant.

Results

Effects of Aaptamine on 3T3-L1 Cell Viability

To establish the toxicity effect of aaptamine on 3T3-L1, we conducted MTT assay. We used dosages at 0, 5, 10, 25, 50, and 100 μM. Our result showed that aaptamine did not harm 3T3-L1 cells when used at doses up to 50 μM but significantly decreased 3T3-L1 cell viability at higher dosage (100 μM) (Figure 2), therefore, aaptamine dosages up to 50 μM were used for subsequent experiments. After non-toxicity dosages are established, we elaborate the effect of aaptamine on 3T3-L1 adipogenesis. We analyzed fenotipe (i.e lipid accumulation using Oil Red O staining), transcriptomic (evaluation of mRNA expression of *Pparg* and *Slc2a4*), and epigenetic (methylation DNA evaluation on promoter of *Pparg*).

Effects of Aaptamine on Lipid Accumulation in 3T3-L1 Adipocytes

Lipid accumulation was analyzed macroscopically and microscopically (Figure 3), showing the dose-dependent effect of aaptamine on lipid accumulation with the least lipid accumulation observed in the 50 μM aaptamine-treated cells. The quantitative analysis of lipid accumulation revealed significant differences (p<0.01) between the treatment groups (Figure 4).

Effects of Aaptamine on Pparg and Slc2a4 mRNA Expression

Pparg mRNA expression was evaluated on day 13 to define the differentiation process (Figure 5), showing that aaptamine dose-dependently reduced *Pparg* mRNA expression. The antiadipogenic effects of aaptamine were assessed by determining its impact on *Slc2a4* mRNA expression (Figure 6), demonstrating that aaptamine also dose-dependently decreased *Slc2a4* expression in 3T3-L1 adipocytes.

Effects of Aaptamine on Pparg Methylation

Pyrosequencing revealed that aaptamine treatment of 3T3-L1 adipocytes had no significant effects on the DNA methylation of the *Pparg* promoter (Figure 7).

Discussion

The energy balance is significantly influenced by adipose tissue which controls lipid metabolism and glucose homeostasis.[21] Consequently, adipose tissue dysregulation will result in obesity-related metabolic and cardiovascular

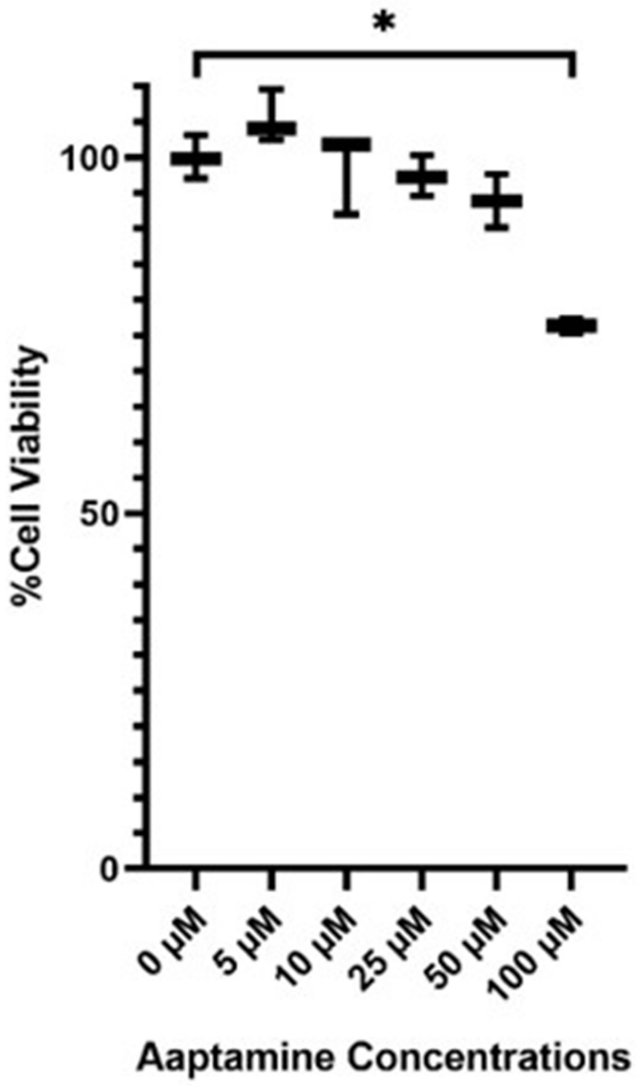

Figure 2 Effects of aaptamine on 3T3-L1 adipocyte viability. Cell viability was assessed by the MTT assay and the experiments were conducted in triplicate. Data are presented as median, maximum, and minimum.*$p<0.05$ according to the Kruskal Wallis test.

diseases.[22] The incidence of obesity and metabolic illnesses has rapidly increased due to the isolation at home during the COVID-19 pandemic.[23,24]

Obesity is linked to lipid buildup, lipolysis, and adipocyte differentiation, regulated by transcription factors like PPARγ and C/EBPα.[25] The *Slc2a4* gene, encoding GLUT4, plays a role in lipogenesis and insulin sensitivity. *Pparg* and *Slc2a4* jointly influence adipose cell differentiation, with *Pparg* acting as a key regulator of adipogenesis and *Slc2a4* facilitating glucose transport in adipocytes.[26,27] *Pparg* may also regulate *Slc2a4*, connecting adipogenesis to lipid metabolism. Additionally, *PPARG*, influenced by lifestyle and diet, regulates metabolism-related genes like *SLC2A4*.[28] This study explored aaptamine's effects on *Pparg* and *Slc2a4* expression, lipid accumulation, and epigenetic changes in 3T3-L1 preadipocytes. Aaptamine inhibited adipogenesis by reducing lipid accumulation and downregulating *Pparg* and *Slc2a4* expression without causing cytotoxicity.

However, there was no apparent effect of aaptamine on the methylation of the *Pparg* promoter during 3T3-L1 adipocyte differentiation. DNA methylation is not the only epigenetic mechanism that can change gene expression, other mechanisms include posttranslational histone modification. Previous studies have shown that histone methylation can inhibit *Pparg* expression which is associated with chromatin structure assembly. High levels of repressive histone methylation, for

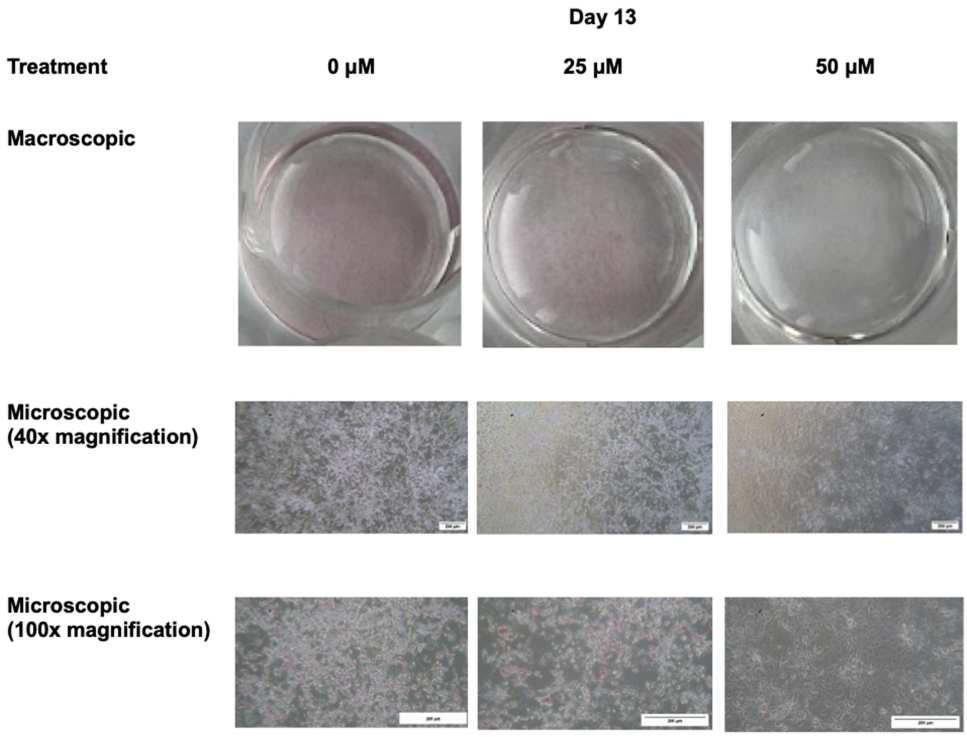

Figure 3 Macroscopic and microscopic features after Oil Red O staining of 3T3-L1 cells treated with aaptamine. (**a**) macroscopic presentation; (**b**) 40x magnification, and (**c**) 100x magnification.

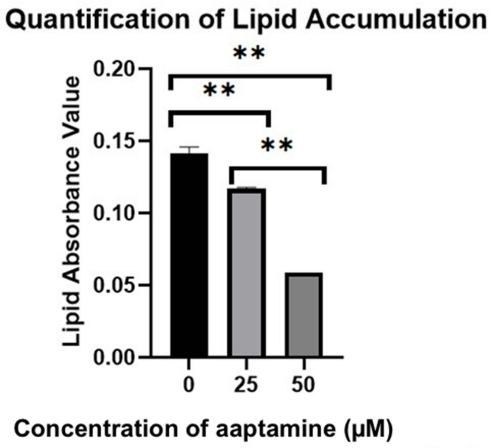

Figure 4 Semi-quantitative analysis of oil Red O stained 3T3-L1 adipocytes treated with aaptamine. Data are presented as mean ± SD. **$p<0.01$ as indicated by ANOVA followed by Tukey's HSD.

example, H3K9me2 and H3K9me3, may cause the DNA wrapped around histones to become more tightly compacted, repressing transcription of *Pparg* and is also often correlated with gene silencing.[29] Recent studies have revealed that histone acetylation can also suppress gene expression and is associated with heterochromatin assembly. High levels of H4K20ac have been found in the transcription start regions of poorly expressed and inactive genes, thus the presence of H4K20ac is associated with very low or no gene expression.[30] Kaimori et al reported that lysine 20 on histone H4, a major methylation site, can also undergo acetylation, indicating that most transcription activators do not bind to genes with high levels of H4K20ac. Conversely, the transcription repressor NRSF/REST is often found in regions rich in H4K20ac, indicating that H4K20ac is a distinct acetylation marker associated with gene expression suppression.[31]

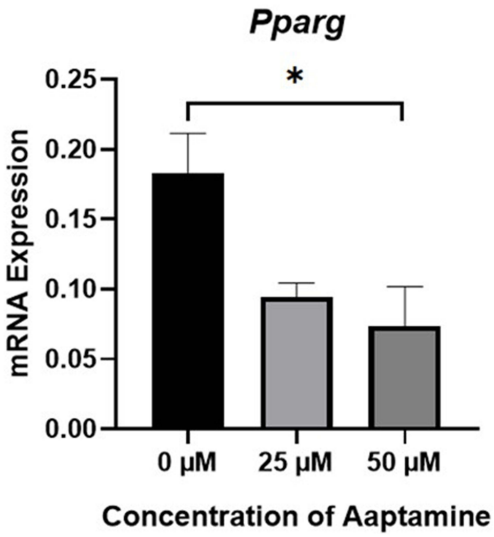

Figure 5 Aaptamine dose-dependently decreased *Pparg* mRNA expression. Experiments were conducted in triplicate and data are presented as mean ± SD. *$p<0.05$ according to ANOVA followed by Tukey's HSD.

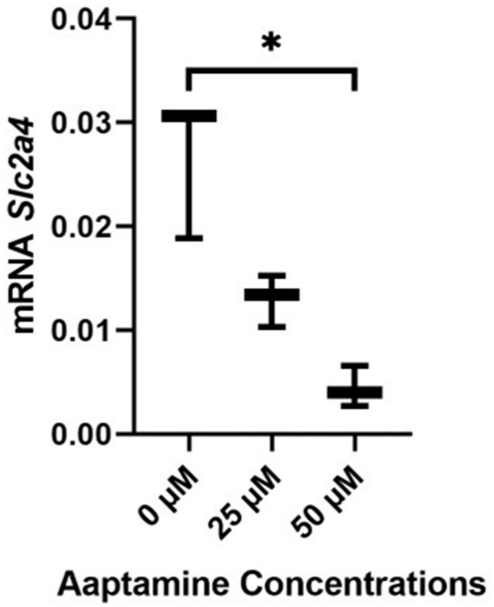

Figure 6 Aaptamine dose-dependently decreased *Slc2a4* mRNA expression in 3T3-L1 adipocytes. Experiments were conducted in triplicate and data are presented as median, maximum, and minimum. *$p<0.05$ according to the Kruskal Wallis test.

In addition to being regulated by DNA methylation and histone modifications, *Pparg* expression during adipogenesis is also controlled by microRNAs (miRNAs) which suppress gene expression by inhibiting translation or reducing mRNA stability.[32] miRNA profiling shows that miRNA expression is depot-specific in adipose tissues,[33] with miRNAs such as miR-27a/b and miR-302a being negative regulators of adipocyte differentiation by directly targeting *Pparg*. Meanwhile, miR-143 indirectly targets *Pparg* activity through its effects on the MAPK signaling pathway.[34–38] miR-143 targets MAP2K5, a kinase that phosphorylates and induces ERK5, a modulator of PPARγ.

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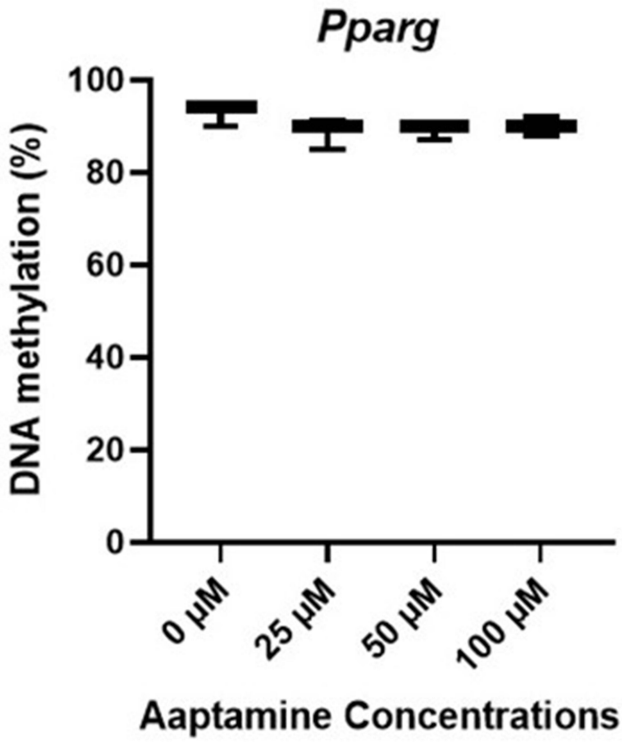

Figure 7 *Pparg* promoter methylation. Experiments were conducted in triplicate and data are presented as median, maximum, and minimum. $p>0.05$ according to the Kruskal Wallis test.

DNA and histone methylation and histone acetylation are closely related and can both be influenced by dietary intake. For example, Basset et al found that quercetin, a flavonoid commonly found in plants, acts as a histone acetyltransferases (HAT) inhibitor. Quercetin can affect histone acetylation directly by inhibiting HAT, meaning the compound directly influences histone acetylation without altering DNA methylation. Quercetin can reduce histone acetylation and influence gene expression by inhibiting HAT activity.[39] Therefore, the effects of aaptamine in reducing *Pparg* mRNA expression and decreasing lipid accumulation are likely caused by mechanisms other than DNA methylation, such as histone modifications and non-coding RNA-mediated gene regulation.

Conclusion

Our findings indicate that aaptamine suppresses adipocyte differentiation by downregulating *Pparg* and *Slc2a4* mRNA expression, subsequently reducing lipid accumulation, suggesting its potential involvement in adipogenesis regulation.

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Disclosure

The authors report no conflicts of interest in this work.

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