ANTI-ADIPOGENIC ACTIVITY OF 4,5,4'-TRIHYDROXY-3,3'-DIMETHOXYBIBENZYL FROM *DENDROBIUM ELLIPSOPHYLLUM* IN 3T3-L1 PREADIPOCYTES

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Pharmaceutical Sciences and Technology
FACULTY OF PHARMACEUTICAL SCIENCES
Chulalongkorn University
Academic Year 2022

ฤทธิ์ยับยั้งการพัฒนาเป็นเซลล์ไขมันของ 4,5,4'-ไตรไฮดรอกซีไบเบนซิล-3,3'-ไดเมทอกซีไบเบนซิลจากเอื้องทองในเซลล์ตั้งต้นไขมันชนิด 3ที3-แอล1

น.ส.นีน เอ เอ คีน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเภสัชศาสตร์และเทคโนโลยี ไม่สังกัดภาควิชา/เทียบเท่า
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2565

Thesis Title	ANTI-ADIPOGENIC ACTIVITY OF 4,5,4'-TRIHYDROXY-3,3'-DIMETHOXYBIBENZYL FROM *DENDROBIUM ELLIPSOPHYLLUM* IN 3T3-L1 PREADIPOCYTES
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นีน เอ เอ คีน : ฤทธิ์ยับยั้งการพัฒนาเป็นเซลล์ไขมันของ 4,5,4'-ไตรไฮดรอกซีไบเบนซิล-3,3'-ไดเมทอกซีไบเบนซิลจากเอื้องทองในเซลล์ตั้งต้นไขมันชนิด 3ที3-แอล1. (ANTI-ADIPOGENIC ACTIVITY OF 4,5,4'-TRIHYDROXY-3,3'-DIMETHOXYBIBENZYL FROM *DENDROBIUM ELLIPSOPHYLLUM* IN 3T3-L1 PREADIPOCYTES) อ.ที่ปรึกษาหลัก : รศ. ภก. ดร.ฉัตรชัย เชาว์ธรรม, อ.ที่ปรึกษาร่วม : ผศ. ดร.เอกชัย พรหมเพชร

โรคอ้วนอุบัติขึ้นทั่วโลกอย่างแพร่หลายและกลายเป็นสิ่งคุกคามสุขภาพของมวลมนุษย์ ในขณะที่เภสัชบำบัดโดยการประยุกต์ใช้ผลิตภัณฑ์ธรรมชาติยังอยู่ในระหว่างการพัฒนา งานวิจัยนี้ได้พิสูจน์บทบาทของ 4,5,4'-ไตรไฮดรอกซีไบเบนซิล-3,3'-ไดเมทอกซีไบเบนซิล (ทีดีบี) ที่สกัดได้จากกล้วยไม้เอื้องทองต่อพัฒนาการและการทำงานของเซลล์ตั้งต้นไขมันจากหนู (3ที3-แอล1) และมนุษย์ (พีซีเอส-210-010) และประยุกต์ใช้เพื่อควบคุมโรคอ้วน ระดับความเป็นพิษต่อเซลล์ไขมันทั้งสองชนิดของทีดีบี(ความเข้มข้นของสารที่มีฤทธิ์ยับยั้งได้ครึ่งหนึ่งของสูงสุด คือ 36 ไมโครโมลาร์) มีค่าสูงกว่าความเป็นพิษของสารดังกล่าวต่อเซลล์ตั้งต้นไขมัน(ความเข้มข้นของสารที่มีฤทธิ์ยับยั้งได้ครึ่งหนึ่งของสูงสุด คือ 72 ไมโครโมลาร์) ถึงสองเท่าและไม่พบความแตกต่างอย่างมีนัยสำคัญของระดับความเป็นพิษต่อเซลล์ไขมันจากหนูและมนุษย์ ทีดีบีสามารถลดการสะสมไขมันและส่งเสริมการย่อยสลายไขมันในเซลล์ทดสอบทั้งสองชนิดทั้งในระยะเริ่มต้นและระหว่างพัฒนาไปเป็นเซลล์ไขมัน โดยประสิทธิภาพจะเพิ่มสูงขึ้นเมื่อเพิ่มความเข้มข้นของทีดีบี การศึกษาในระดับชีวโมเลกุลพบว่าทีดีบีสามารถหยุดการเปลี่ยนผ่านของเซลล์ตั้งต้นไขมันในระยะ จี0/จี1 ของวัฏจักรเซลล์โดยการควบคุมตัวกระตุ้น และตัวยับยั้งต่างๆ ในกระบวนการเพิ่มปริมาณเซลล์ที่มีคุณลักษณะเดียวกัน สารดังกล่าวยังสามารถควบคุมการพัฒนาเป็นเซลล์ไขมันผ่านการยับยั้งการแสดงออกของตัวควบคุมและตัวส่งผลในกระบวนการพัฒนาเป็นเซลล์ไขมัน โดยการยับยั้งวิถีสัญญาณเอเคที/จีเอสเค-3เบต้าและกระตุ้นวิถีเอเอ็มพีเค-เอซีซี ทีดีบีได้แสดงให้เห็นถึงศักยภาพทางเภสัชกรรมในการควบคุมพัฒนาการและการทำงานของเซลล์ไขมัน และเป็นสารทางเลือกที่มีศักยภาพในการพัฒนาผลิตภัณฑ์ธรรมชาติเพื่อใช้เป็นยาบำบัดรักษาโรคอ้วนต่อไป

สาขาวิชา	เภสัชศาสตร์และเทคโนโลยี	ลายมือชื่อนิสิต .....
ปีการศึกษา	2565	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
		ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

6273006433 : MAJOR PHARMACEUTICAL SCIENCES AND TECHNOLOGY

KEYWORD: Dendrobium ellipsophyllum, bibenzyl, adipocyte differentiation, obesity

Hnin Ei Ei Khine : ANTI-ADIPOGENIC ACTIVITY OF 4,5,4'-TRIHYDROXY-3,3'-DIMETHOXYBIBENZYL FROM *DENDROBIUM ELLIPSOPHYLLUM* IN 3T3-L1 PREADIPOCYTES. Advisor: Assoc. Prof. CHATCHAI CHAOTHAM, Ph.D. Co-advisor: Asst. Prof. EAKACHAI PROMPETCHARA, Ph.D.

Obesity and its global prevalence have become a threat to human health due to not only the abnormal lipid accumulation in the body as a result of aberrant expression levels in adipocyte differentiation and lipogenesis but also the underdeveloped pharmacotherapy. Recently, the bioactive compounds from the natural products have gained a lot of attention due to undertaking prophylactic and therapeutic effects on obesity. Here, we probed how 4,5,4′-trihydroxy-3,3′-dimethoxybibenzyl (TDB) extracted from an Thai orchid (*Dendrobium ellipsophyllum*) could exert its roles on the differentiation and function of murine (3T3-L1) and human (PCS-210-010) preadipocytes and offer some implications to modulate obesity. Cytotoxic effects of TDB on preadipocytes (Half maximum inhibitory concentration; IC_{50} = ~36 μM) were two-fold higher than those detected on adipocytes (IC_{50} = ~72 μM), and no significant difference was detected in cytotoxic profiles between both cells. TDB in a dose-dependent manner decreased cellular lipid accumulation and enhanced lipolysis of both cell lines assessed at early differentiation and during maturation. Underlining molecular mechanisms proved that TDB paused the cell cycle progression by regulating inducers and inhibitors in mitotic clonal expansion, leading to growth arrest of preadipocytes at the G0/G1 phase. The compound also governed adipocyte differentiation by repressing expressions of crucial adipogenic regulators and effectors through deactivating the AKT/GSK-3β signaling pathway and activating the AMPK-ACC pathway. To this end, TDB has shown its pharmaceutical potential for modulating adipocyte development and function, and it would be a promising candidate for further assessments as a therapeutic agent to defeat obesity.

Field of Study:	Pharmaceutical Sciences and Technology	Student's Signature .....
Academic Year:	2022	Advisor's Signature .....
		Co-advisor's Signature .....

ACKNOWLEDGEMENTS

First and foremost, I would like to deeply thanks to my academic advisor, Assoc. Prof. Chatchai Chaotham, Ph.D. for accepting into his research group and contributing to a rewarding high quality graduate school experience by giving me his invaluable mentorships and guidance during my tenure. I also would like to extend my gracious thanks to my academic co-advisor, Asst. Prof. Eakachai Prompetchara, Ph.D. for his valuable assistances and kind suggestions from his keen scientific insight. Additionally, I would like to special thanks my thesis committee members: Asst. Prof. Monruedee Sukprasansap, Ph.D., Assoc. Prof. Boonchoo Sritularak, Ph.D., Asst. Prof. Preedakorn Chunhacha, Ph.D., and Asst. Prof. Natapol Pornputtapong, Ph.D. for their valuable suggestions, encouragement, and interest in my work. I also wholeheartedly thanks to Prof. Dr. Christian Pilarsky of Friedrich-Alexander-University (FAU) Erlangen-Nürnberg and Universitätsklinikum Erlangen, Germany for hosting me as a research fellow to do the organoids training in the laboratory for six months. I am grateful for the funding sources that allowed me to pursue my graduate school studies: (1) Scholarship for International Graduate Students in ASEAN and Non-ASEAN Countries, Chulalongkorn University, (2) Overseas Research Experience Scholarship for Graduate Students, Chulalongkorn University, (3) The 90th Anniversary of Chulalongkorn University under Rachadapisek Somphot Fund, and (4) the research fund from the Faculty of Pharmaceutical Sciences, Chulalongkorn University (Grant No. Phar2563-RG006). I also would like to give my sincere gratitude to all teachers and members of Pharmaceutical Sciences and Technology program, Faculty of Pharmaceutical Sciences, Chulalongkorn University and Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, for helping me in numerous ways and giving me many wonderful learning experiences. I also specially thank the lab members for their assistance and helpfulness. Moreover, I would like to credit the graduate officers and international students' officers of Faculty of Pharmaceutical Sciences, Chulalongkorn University for providing much needed assistance with administrative tasks, reminding us of impending deadlines, and keeping our work running smoothly. Last of all, I also would like to thank my parents, relatives, friends, and all the people who contributed mental and physical encouragements and supports to be able to finish this milestone.

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CHAPTER I

BACKGROUND AND RATIONAL

Although being a non-communicable disease, obesity represents as a serious risk factor for various metabolic disorders, including hyperglycemia, hyperlipidemia, hypertension, and cardiovascular diseases resulting in socioeconomic burden and increased mortality rate. The rising prevalence of obesity is an important priority in clinical field to limit the obesity related metabolic consequences [1, 2]. Obesity is characterized with the excessive growth and expansion of adipose tissue via increase in both number and size of differentiated adipocytes whereby adipocytes play a decisive role in regulation of lipid homeostasis contributing to obesity [3, 4]. Appropriately, the concept of inhibition of obesity through halting the differentiation of adipogenic progenitor cells and/or triggering the decomposition of accumulated lipid droplets in adipocytes has attracted a lot of research attentions in recent years [5, 6]. Although anti-obesity medication is an effective therapeutic approach for obesity related metabolic disorders, most prescribed drugs have unfavorable side effects [7].

Adipogenesis, also known as adipocyte differentiation, is the conversion process from undifferentiated preadipocytes to mature adipocytes [8]. Adipogenesis is regulated through complex cascades, including the coordinated changes in hormonal sensitivity and gene expressions accompanied with morphological alteration [9]. After induced to differentiation, growth-arrested preadipocytes synchronously reenter into cell cycle and initiate the mitotic clonal expansion (MCE) evidenced by down-regulation of cyclin-dependent kinase inhibitor (CDKI) and cyclin D1 as well as overexpression of cyclin D3 and cyclin-dependent kinase (CDK) [10, 11]. Subsequently, the cell cycle is terminated to instigates the early phase of differentiation, which is involved with sequential changes in gene expressions [12].

The activation of Protein kinase B (Akt) is essential for cell survival, cell cycle progression, differentiation and adipogenesis prior entering to MCE [13]. Activated Akt (p-Akt) modulates the activity of glycogen synthase kinase 3β (GSK3β) [14] and downregulates the phosphorylation of 5′-adenosine monophosphate-activated protein kinase (AMPK) [15], which in turn reduces the phosphorylated acetyl-CoA carboxylase (p-ACC) to contribute the adipocyte differentiation [16, 17]. Intriguingly, inactivated GSK3β (p-GSK3β) and suppression of AMPK/ACC signal are correlated with up-regulation of adipogenic transcription factors mediated by Akt (Ref). Moreover, p-Akt inactivates the function of CDKI, including p18, p21 and p27 consequence with the formation of cyclins-CDK complex, implying for the progression of MCE and inauguration of early stage of adipocyte differentiation [18]. Evidently, Akt has been proposed as a targeted molecule for inhibiting adipogenesis [19].

At the early stage, adipogenesis is mediated by adipogenic transcription proteins of peroxisome proliferator-activated receptorγ (PPARγ), CCAAT/enhancer-binding proteinα(C/EBPα) and sterol regulatory element binding protein-1 (SREBP1) [19, 20]. PPARγ is the center of transcription factor that is responsible for initiating differentiation and formation of adipocytes, while C/EBPα and SREBP1 crucially facilitate lipid accumulation to complete adipogenic program [21, 22]. Additionally, these transcription factors augment intracellular triglyceride level in mutual adipocytes through modulating the expression of fatty acid synthase (FAS), fatty acid binding protein 4 (FABP4), lipoprotein lipase (LPL) and perilipin 1 (PLIN1) [23, 24].

As a resource of safe and potent therapeutic compound, there have been increasing motivation in natural bioactive compounds-based research to inhibit adipogenesis and lipid accumulation for obesity treatment [25, 26]. A plant bibenzyl of 4,5,4′-trihydroxy-3,3′-dimethoxybibenzyl (TDB) extracted from a Thai orchid,

Dendrobium ellipsophyllum has been reported for selective anti-cancer activity against human lung cancer cells via suppression on Akt signal [27, 28]. However, the inhibitory effects of TDB on adipogenesis and lipogenesis and related mechanisms has not been evaluated. It would be worthwhile to investigate the potential anti-adipogenic activity of TDB in 3T3-L1 preadipocytes whereby the present study also confirmed not only anti-adipogenic effect but also lipolytic effect of TDB in human preadipocyte PCS-210-010 cells. The acquire information would facilitate the development of this bibenzyl from *D. ellipsophyllum* as a safe and effective therapy for obesity.

CHAPTER II

LITERATURE REVIEW

2.1. Obesity

In recent years, obesity is becoming alarmed to the public health due to witnessing in increased socioeconomic burden and increased morality rates not only in developing countries but also in developed countries [28, 29]. The world population approximately 37% of adult and 14% of children are dealing with overweight or obesity and 3.4 millions of people are annually died caused by obesity at which there are no countries succeeding in reduction of obesity [30]. Despite obesity being non-communicable disease, clinically important level is large due to perilously exacerbate in the health consequences and alarming the risk of death confirmed by epidemiological studies and life-insurance data [1, 2, 31]. Moreover, it is related with dramatic increase in serious risk factors for various obesity-mediated metabolic diseases including hyperglycemia, hypertension, cardiovascular diseases, certain forms of cancer, arthritis, and respiratory complications [32, 33]. Thus, tackling the rising prevalence of obesity is an important priority in clinical field to limit the obesity stimulated metabolic complications [34, 35].

2.1.1. Measurement of obesity

According to WHO data, body mass index (BMI) is a common indicator to roughly predict the obesity in populations although it doesn't directly measure the body fat. It is based on height and weight of the body at which weight (kg) of a person is divided by height (m^2) to get the BMI. The BMI level can help to find out the healthy and unhealthy body conditions. Although the ideal BMI depends on race, the BMI ranged lower or higher than 18-25 have normally been related with risks of metabolic consequences whereas BMI level greater than 25 is defined as obesity [30].

2.1.2. Current treatment for obesity

Although prevention through changes in obesogenic lifestyles is long-term goals, medical treatment is compulsory for those with already obese conditions [36, 37]. Surprisingly, there are quite limited options for obesity treatment. Bariatric surgery including sleeve gastrectomy, gastric bypass, and gastric banding are effective in weight loss, however, its surgical complications tend to prone the unhealthy life consequences [38, 39].

Therapeutic agents are the alternative strategy to treat obesity at which body weight is reduced through reduction of consumption, decreasing the food absorption, and increasing the energy expenditure [40]. Orlistat is an anti-obesity agent which effectively reduces the absorption of food intakes and the body weight [41]. In addition, liraglutide and exenatide are the therapeutic anti-obesity agents which suppress the appetite resulting in weight loss [42]. Furthermore, hormonal based therapeutics such as topiramate and phentermine are used for obesity treatment [43]. Unfortunately, even with only a handful of anti-obesity agents are permitted with license for clinical use, the currently marked anti-obesity agents still cannot pursue for long term success and trigger the undesirable side effects, including increase blood pressure and heart rate, insomnia, and liver damages [44, 45]. Thus, new treatments with well tolerance, good efficacy and low side effects are urgently required to tackle the obesity and obesity-mediated complications [46].

2.2. Alteration of adipose tissue in obesity

Obesity is characterized with an excessive growth and expansion of adipose tissue mass through increases in both the number and size of differentiated adipocytes. Adipocytes play a decisive role in regulation of lipid homeostasis

contributing to obesity [47]. As the site of fat storage, excess net gain of calories through food intake causes abnormally accumulation of fats in adipose tissue and produce the body condition called obesity while the moderate storage of fat in adipose tissue is desirable for physiological sense. Thus, dysfunction in adipose tissue is correlate with obesity-induced metabolic impairment [48, 49].

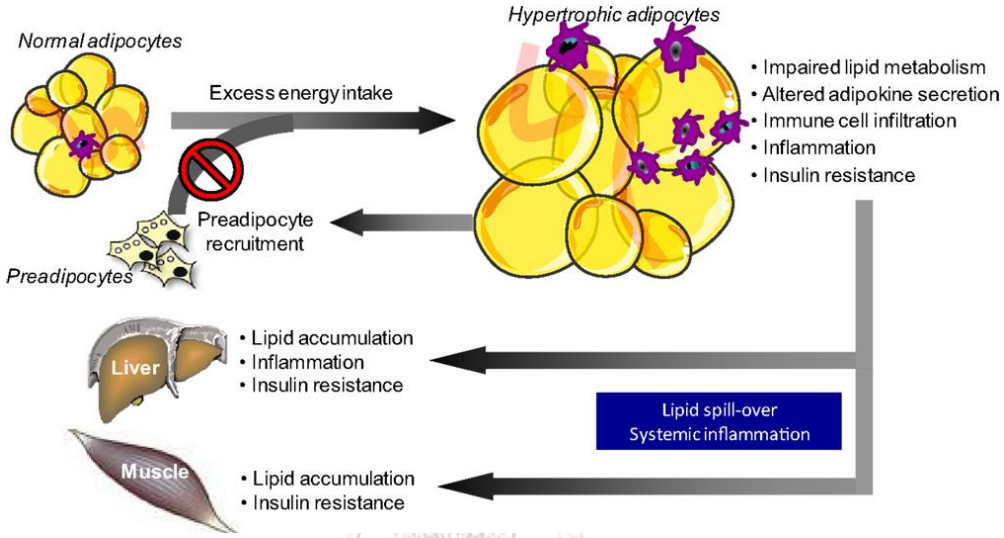


Figure 1. Obesity mediated adipocyte dysfunction and metabolic consequences [25]

Generally, preadipocytes differentiate into normal adipocytes and participate in normal energy homeostasis required for body functions. During positive energy balance, there is a dynamic mechanism to reorganize the adipose tissues at which adipocytes secrete cytokines and hormones to facilitate the recruitment of preadipocytes. This process is known as adipose tissue remodeling. However, long-term positive energy intake induces the weight gain and triggers the normal adipocytes to adipocyte hypertrophy accompanied with impaired lipid metabolism, altered adipokine and inflammatory cytokine secretions which in turn diminish the preadipocyte recruitments leading to worsening of obesity. Together, dysregulation of lipid metabolism and adipogenic regulation secretions not only increase the insulin resistance but also cause detrimental effects in the whole body especially at liver and muscle through the lipid spillover in the circulation contributing to the

development of obesity mediated chronic diseases and insulin resistance (Fig. 1) [50]. Appropriately, the concept of inhibition of obesity through halting the differentiation of adipogenic progenitor cells and/or triggering the decomposition of accumulated lipid droplets in adipocytes has attracted a lot of research attentions in recent years [51].

2.3. Adipose tissue

Adipose tissue is exemplified with its cellular heterogeneity and among its cellular components, adipose tissue is majorly composed with preadipocytes, adipocytes, endothelial cells, fibroblasts, and multipotent stem cells which in turn differentiate into variety of cell types. Thus, adipocyte tissues can be alternatively named as fat tissues due to consisting of approximately one-third of the mature adipocytes. The remaining parts are a mixture of endothelial precursor cells, T regulatory cells, macrophages, small mesenchymal stem cells (MSCs), and preadipocytes of different stages [52, 53]. Preadipocytes can proliferate or differentiate into mature adipocytes, presenting the adipose tissue as a constant functional plasticity. Adipocytes are the fat cells or lipocytes and can be found at the stereotypical depots of whole body [54].

2.3.1. Types of adipose tissue

There are two major types of adipose tissues, namely brown adipose tissue (BAT) and white adipose tissue (WAT), both of which possess a few different significant properties [55]. Brown adipocytes are characterized as multilocular lipid droplets, high mitochondrial content with nucleus position around at the center of the cells while white adipocytes are characterized with single, large lipid droplets with low mitochondrial content and nucleus is also at the cell membrane border. White adipocytes are extensively distributed throughout the body and its main functions is to

regulate the body's lipid homoeostasis, whereas brown adipocytes are relatively scarce, and its major role is to provide the body heat and it is essential during newborn and infants [55, 56]. Therefore, white adipocytes play a major role in energy reservation, and it stores the energy as the triacylglycerol (TG) during energy excess and releases the energy as free fatty acids during energy deficiency [57].

2.3.2. Different between hyperplasia and hypertrophy of adipocytes

Increase in adipocyte number and increase in adipocyte volume are known as hyperplasia and hypertrophy, respective and both conditions are characteristics of obesity [58, 59]. Overall increase in weight is correlated with certain degree of hypertrophy and obesity severity in obese individual is strongly correlated with hyperplasia. Prolonged excess weight gain and increased size of adipocytes precede in development of adipocyte number [50]. Additionally, sustained hypertrophy of adipocytes results in diabetogenic especially increased risk of type 2 diabetes [47]. It is also documented that consequence of both increased adipocyte hyperplasia and hypertrophy give rise to the progressing of obesity [60, 61].

2.4. Adipose stem cells

Preadipocytes are kind of adipose stem cells and situated in the adipose tissue at the part of stromal vascular fraction (SVF). Proliferation and differentiation of preadipocytes are mutually exclusive processes. Nonetheless, both events are closely regulated to establish at the early phase of adipocyte differentiation [62, 63]. As shown in figure 2, fully confluence preadipocytes trigger the growth arresting and hormonal induction during that time will encourage the re-entry of cell cycle or mitotic clonal expansion to finally undergo the terminal differentiation into adipocytes, implying that there is the crosstalk existed between mechanism of cell

proliferation and factors influencing cell differentiation. This knowledge allows in better understanding of development of adipocytes during physiological and pathophysiological conditions and might assist in creating therapeutic agents for prevention and treatment of obesity [64]. For metabolic and obesity studies, preadipocytes are cultured and induced to differentiation conferring to adipocytes [60, 61].

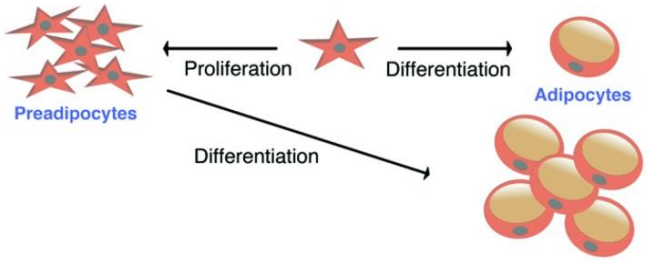


Figure 2. Cross-talk between preadipocyte proliferation and differentiation [39]

2.5. Adipocyte differentiation

Adipocyte differentiation, also known as adipogenesis is the conversion process from undifferentiated preadipocytes to differentiated adipocytes. It is regulated through a complex process including the coordinated changes in hormonal sensitivity and gene expressions accompanied with morphological changes [7, 8]. These sequential changes are reflected not only with the lipid or triglyceride accumulation but also with the appearance of gene or protein markers at various stages including mitotic clonal expansion (MCE), early, intermediate, and late differentiation stages [17, 21]. During adipogenic differentiation, several transcription markers regulate the level of adipogenesis and create the phenotype of adipocyte [65]. Moreover, cytoskeletal components, cell morphology, and level of extracellular matrix (ECM) secretions are obviously changed during adipocyte differentiation. A detailed understanding of adipogenesis process on molecular and cellular level is important to maintain the lipid homeostasis for therapeutic purpose and to regenerate the adipose tissue for cosmetic purpose [66, 67].

2.5.1. Mitotic clonal expansion at the early phase of adipocyte differentiation

Among the cascade of events to undergo adipogenesis, mitotic clonal expansion (MCE) is the one of the earliest processes and it plays as the pivotal step for adipocyte differentiation cascade [21]. Figure 3 demonstrates that fully confluent preadipocytes halt the cell proliferation through physical contact inhibition with neighboring cells after two days of culturing prior to initiate the differentiation (Day 2). Growth arrested cells reenter into the cell cycle and undergo several rounds of cell cycle which is considered as MCE. Upon confluence, preadipocytes are ceased of the growth at the G0 phase of cell cycle wherein cyclin-dependent kinase inhibitors (CDKI) such as p18, p21 and p27 are augmented. At the G1 phase of cell cycle, tumor suppressor retinoblastoma (Rb) is hyperphosphorylated and degradation of CDKI instigate to activate and assemble cyclin E to CDK2, and cyclin D with CDK4 and CDK6. Complex of cyclin D with CDK4/CDK6 is the major controller at the G1 phase of cell cycle, while cyclinE-CDK2 complex is an important regulator mainly at the transition point of G1 to synthesis (S) phase [17].

Complex formation between cyclin and CDK are mandatory for its kinase activity, Rb phosphorylation, and regulation of E2F transcription. Hyperphosphorylated Rb proteins changes into hypophosphorylated form to suppress the cell cycle during terminal stage of differentiation [10]. The S phase of cell cycle entry happens approximately 14 h after adipogenic cocktail treatment and the peak DNA synthesis can be observed after about 18 h of hormonal induction [10, 18]. Consequently, inhibition of cell-cycle

progression through suppression of cell-cycle regulators and activation of CDKI during MCE might provide an effective therapeutic target to inhibit adipogenesis and obesity [19].

According to figure 3, preadipocytes maintain the high expression levels of preadipocyte factor-1 (Pref-1). During adipogenesis, peroxisome proliferator-activated receptorγ (PPARγ) and subunits of CCAAT/enhancer-binding proteins (C/EBP) participate in mediation of adipocyte differentiation during early phase while fatty-acid synthase (FAS), adipogenesis related protein 2(aP2) and lipoprotein lipase (LPL) are dominant at terminal stage of differentiation [17].

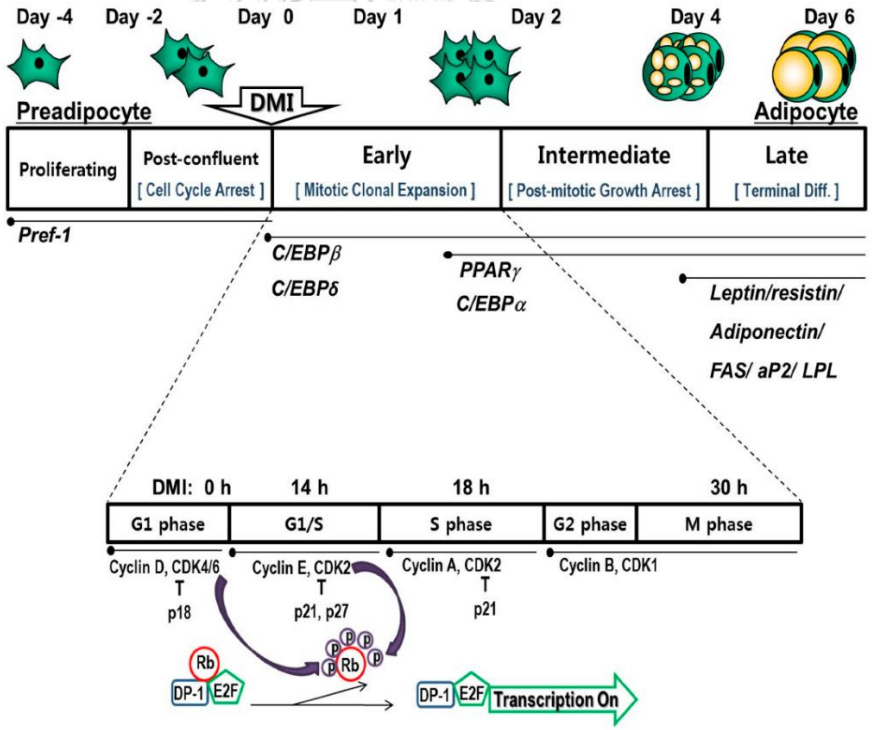


Figure 3. A scheme of adipogenesis from preadipocyte to adipocytes [43]

In addition, cyclins are important for mitotic clonal expansion and adipogenesis, as an instance, recent reports evaluated that increased cyclin D1 and cyclin D3 take parts in delaying of cell cycle progression

resulting in stimulation of adipogenic transcription factor (PPARγ) to induce the adipocyte differentiation [69]. There are reports demonstrating that activation of CDKI (p18, p21, and p27) inhibits the CDKs (CDK2, CDK4, and CDK6) contributing to suppression of MCE and reduction of adipogenesis [70]. Besides, according to previous study, MCE is one of the crucial steps to commence the adipocyte differentiation and it is prerequisite to activate the major adipogenic transcription markers (PPARγ, CEBPα) at the early stage of adipogenesis [21].

2.5.2. Transcription factors participating during adipocyte differentiation

During adipocyte differentiation, extracellular hormonal inductions stimulate the glucocorticoid receptor pathway and augment the expressions of adipogenic transcription regulators including PPARs, C/EBPs and SREBPs to progress the differentiation events [71]. In detail, fully growth-arrested cells are stimulated with adipogenic cocktail including isobutylmethylxanthine (IBMX), dexamethasone (DEX) and insulin to activate the adipogenic transcriptors [72]. DEX is a synthetic glucocorticoid which degrades the Pref-1 to initiate the upregulation of glucocorticoid pathway and elevation of C/EBP expressions [72, 73]. Moreover, insulin acts on the insulin like growth factor 1 (IGF-1) receptor [74]. Meanwhile, IBMX, a major regulator, acts as a phosphodiesterase inhibitor to induce the cAMP-dependent protein kinase (AMPK) pathway and it also participates in activation of C/EBP expressions [75]. Furthermore, fetal bovine serum (FBS), used in culture media, takes part in rapid formation of adipogenic phenotype [65]. The members of transcription factors including PPARγ, C/EBPα and SREBP1 play a crucial role during the early phase of adipogenesis. These transcription markers not only terminate the MCE but also induce the lipid

metabolism mediated genes to involve in maintaining of adipocyte phenotype and accumulation of intracellular lipid droplets (Fig. 4) [76].

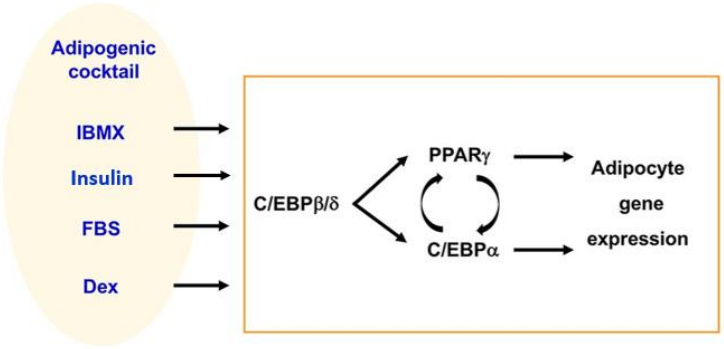


Figure 4. Effect of adipogenic cocktail on transcription of adipogenesis [57]

As demonstrated in figure 5, PPARγ, C/EBPα and SREBP1 transcription factors are particularly involved in regulation of the anti-mitotic activity and development of adipocyte differentiation. Firstly, PPARγ is the main regulator for adipogenic programming which is essential not only for maintaining of the differentiated state but also for adipogenesis. Besides, PPARγ collaborates with CEBPα to regulate the expressions of enzymes involved in triglyceride synthesis, resulting in regulation of genes involved in lipid metabolism, including FAS, LPL and perilipin1 (PLIN1) and promotion of lipid accumulation in adipocytes [77]. Thus, adipocyte-specific PPARγ deleted mice shows the impairment of lipid metabolism through the blocking of high-fat diet (HFD)-stimulated obesity [78]. Secondly, activation of CEBPα ceases the MCE and downstream targeted genes including PPARγ and SREBP1 to control the adipocyte differentiation [77, 79]. Besides, CEBPα knockout mice were reported with depletion of lipid storge in white adipose tissue even though high-fat diet were fed [80]. Lastly, SREBP-1 expressions are important in adipocyte differentiation at which its enhancement activates

the major adipogenic transcriptors (PPARγ) to regulate the differentiation process [81]. Overexpression of SREBP1 increased the adipocyte fat formation and dysregulated the lipid metabolism in 3T3-L1 adipocytes and adipose tissue of mice [82]. The cross-regulation between PPARγ, C/EBPα and SREBP1 gives the positive feedback loops which in turn regulate the downstream signals of lipid metabolism [77].

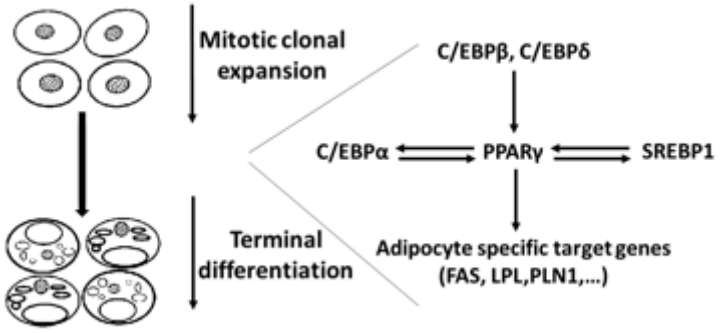


Figure 5. Involvement of transcription factors in adipocyte differentiation [58]

2.5.3. AMPK/ACC regulated adipocyte differentiation

The modulation of AMPK at the early phase of adipogenesis is important for progression of lipid accumulation in adipose tissue and development of obesity due to acting as intracellular energy sensor to regulate the food intake and energy demand inside the body. AMPK activation inhibits the adipogenic transcription markers such as PPARγ and C/EBPα, which suppress the adipogenesis process [83, 84]. As shown in figure 6, activation of phosphorylated AMPK shows the direct positive effect with phosphorylated ACC to impart at adipogenesis and lipid metabolism in the adipose tissue. Moreover, AMPK-mediated ACC activation inhibits the adipogenesis through downregulation of adipogenic markers (PPARγ, C/EBPα and SREBP1-c) [85-87]. AMPK/ACC cascades also regulate the lipid

metabolism through both suppression of lipogenesis and modulation of lipolysis using fatty acid metabolism-associated genes including fatty acid synthase (FAS), fatty acid binding protein 4 (FABP4), and Perilipin (PLIN), eventually resulting in downregulated intracellular lipid droplets accumulation in adipocytes. Furthermore, a recent report shows that AMPK/ACC activation in adipocytes not only inhibited the PPARγ and C/EBPα markers but also counterbalanced the lipid metabolism using FABP4, lipoprotein lipase (LPL) and adiponectin [87].

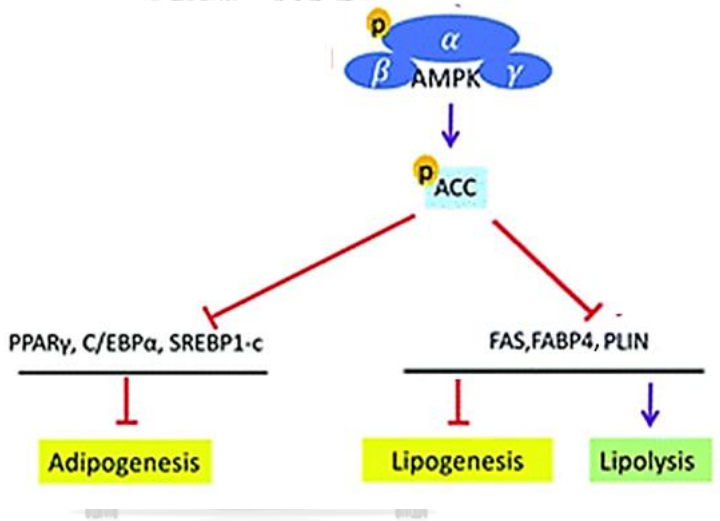


Figure 6. AMPK-modulated adipogenesis and lipid metabolism [68]

Moreover, Akt-mediated GSK3β cascades indirectly augment the adipogenic transcription genes through suppression of AMPK pathway to promote the cellular adipogenesis at the early stage of adipocyte differentiation. The mutual relationship between Akt activation and AMPK deactivation results in the inhibition of phosphorylated acetyl-CoA carboxylase (ACC) to exacerbates the lipogenesis, leading to obesity, as shown in figure 7 [88]. Thus, targeting the AMPK signaling cascades might be potential for suppression of adipogenesis during early phase and regulation of lipid metabolism, which are important for the inhibition of obesity [83].

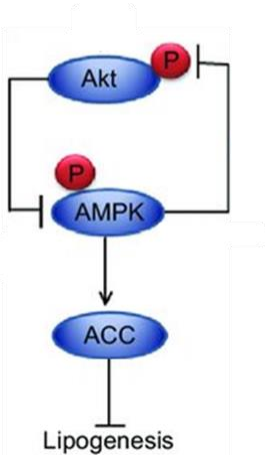


Figure 7. Akt mediated lipogenesis through AMPK pathway in adipocyte [69]

2.5.4. Akt- GSK3β signal in adipocyte differentiation

Upon administering of adipogenic cocktails, Akt/GSK3β pathway is regulated to involve in the cellular proliferation, differentiation, and lipid metabolism [17]. As the Akt being the biological responder of insulin, insulin containing in adipogenic cocktails motivates the insulin receptor substrate 1 (IRS1) which regulates the activation of Akt to phosphorylated form associating with adipogenesis process and de novo lipid metabolism [89, 90]. Activation of Akt pathway indirectly augments the adipocyte differentiation through the limitation of CDKI (p18, p21 and p27) and enhancement of cyclin-CDK complexes through modulation of Rb phosphorylation which are involved in the G1 phase of cell cycle. Thus, activation of Akt cascade contributes the early phase of adipocyte differentiation whereas its deactivation suppresses adipogenesis (Fig. 8) [90].

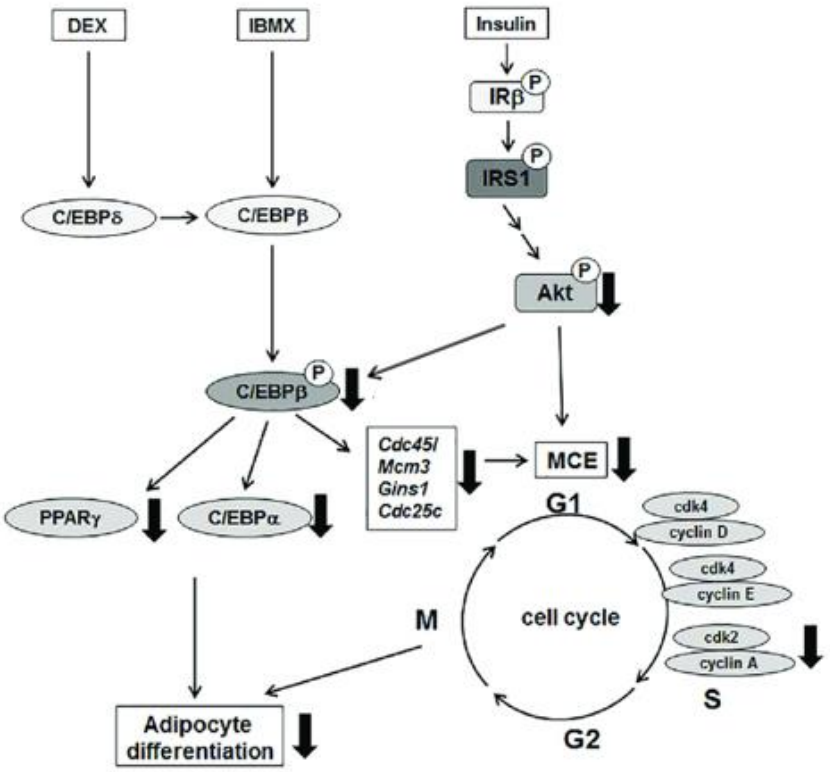


Figure 8. Akt mediates adipocyte differentiation through MCE [71]

Extracellular signals induce the PI3K/Akt pathway in which Akt acts as a positive controller for GSK3β. Interestingly, activation of Akt/GSK3β cascade directly enhances the major adipogenic genes to further undergo the adipocyte differentiation and intracellular lipid accumulation (Fig. 9) [91]. Consequently, recent reports revealed that knock out of Akt gene in HFD-mice were resistant to accumulation of fats in adipose tissue and resulted in reduced expressions of PPARγ and C/EBPα. Therefore, Akt mediated GSK3β pathway is important in differentiation of adipocytes, especially at the early stage of adipogenesis [92, 93].

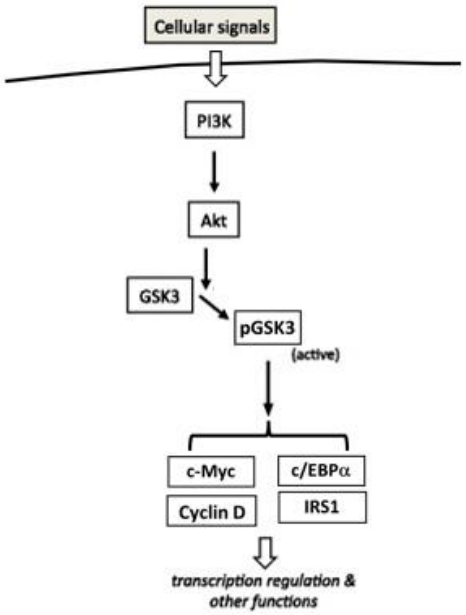


Figure 9. Akt/GSK3 regulates adipogenic transcription pathway [72]

2.5.5. Lipogenesis and lipolytic roles at terminal stage of adipogenesis

Lipid metabolisms, including lipid storage and lipid disintegration impart in crucial role in adipocytes [94]. Typically, increased uptake of fatty acids into the adipocytes causes the excessive deposition of fat in obesity. This process is mediated in adipocytes through expressions of various genes and protein markers, including adipogenic markers (PPARγ, C/EBPα and SREBP1), lipogenic markers (FAS, FABP4, PLIN1) and adipogenic maturity markers (adiponectin and LPL), as shown in figure 10 [21, 95]. FAS and FABP4 are essential factors required for mitochondrial fatty acid oxidation and lipid accumulation in the cytoplasm. LPL is one of the key players for neutral lipid metabolism as it is crucial for storage of triglyceride inside the cells and maintenance of adipose tissue. Adiponectin and PLIN1 are important genes engaged for the growth of lipid loaded adipocytes and regulation of fatty acid metabolism in the adipocytes [21, 96]. Consequently, FAS knock out mice and FABP4 gene deleted mice are resistant to the high-fed diet condition and potential in reduction of obesity and its consequences [78,

97]. The deficiency of LPL in adipose tissue of mice show the reduction of lipid accumulation and counterbalance the lipid metabolism [98]. Besides, adiponectin deleted HFD-mice and PLIN1 knock out HFD-mice show lean body mass with small fat accumulation and enhanced lipolysis [99-101].

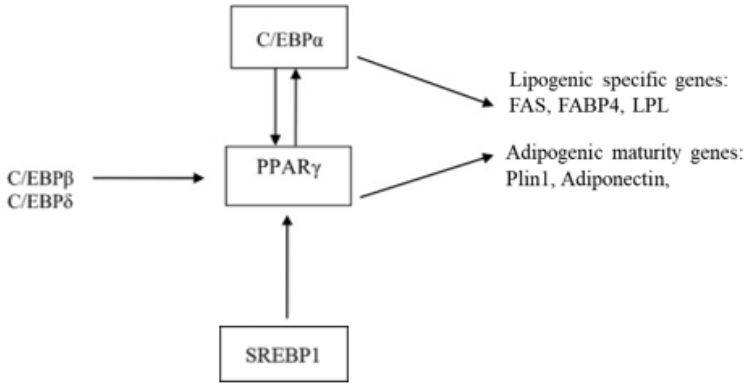


Figure 10. Molecular relationships during lipid metabolism [42]

2.6. Inhibition of adipogenesis by natural bioactive compounds

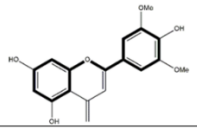
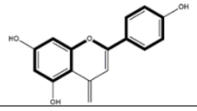
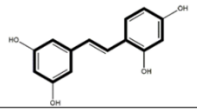
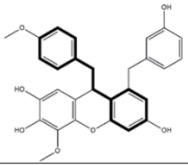
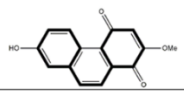
Bioactive compounds derived from natural products exhibit anti-obesity benefits due to influencing effect on fat accumulation and aversion of diet-induced obesity. Moreover, various natural bioactive compounds display its effects on growth suppression of adipose tissue, inhibition of adipocyte differentiation, induction of lipolysis and enhancing of apoptosis in mature adipocytes, thereby diminishing the adipose tissue mass [102]. Bibenzyls, flavonoids, anthocyanins, phenolic acids, and organic acids are the bioactive compounds elucidating the anti-adipogenic, anti-lipogenic and lipolytic effects through various molecular mechanisms [21, 103].

2.6.1. Structure related to anti-adipogenic mechanism

Bioactive compounds generated from natural products have diverse molecular targets to involve in multiple modes of action including adipogenesis, glucose homeostasis, and lipid metabolism to display anti-obesity effects [102]. Table 1 summarizes some examples of antiadipogenic activity related natural bioactive compounds. Several natural bioactive

compounds (flavonoids and stilbenes including stilbenoids, bibenzyls, and phenanthrenes) have been shown to significantly decrease the adipogenic differentiation to evaluate anti-adipogenic activity. However, there has been no reports of anti-adipogenic activity being evaluated during the targeted maturation phase [104-109]. The basic skeleton of the stilbene structure (C6-C2-C6) occurs naturally in several plant species, including *Dendrobium* sp., and has been discovered to possess anti-obesity, anti-cancer, antioxidative, cardioprotective and neuroprotective activities. Moreover, methylated group in stilbene structure are important for compound stability, bioavailability, and bioactivity [110].

Table 1. Natural bioactive compounds with antiadipogenic activity

Bioactive compound	Plants	Differentiation	Maturation	Reference
Tricin (flavonoid) 	Oat hull	Yes	NR	Lee et al., 2015
Genistein (flavonoid) 	Soybean	Yes Yes	NR NR	Valli et al., 2018 Ahmad et al., 2019
Oxyresveratrol (Stilbenoid) 	*Morus alba L.* (mulberry)	Yes	NR	Tan et al., 2015
Dendrofalconerol B (Bibenzyl-phenanthrene) 	*Dendrobium harveyanum*	Yes	NR	Maitreesophone et al., 2022
Densifloral B (Phenanthrene) 	*Dendrobium delacourii*	Yes	NR	Thant et al., 2022

2.6.2. Potential on suppression of the obesity by bibenzyl and its derivatives

Among many bioactive natural products, bibenzyl compounds have been highlighted due to antiobesity potential and safety profile through the reduction of adipocyte differentiation and recovering the loss balance between lipogenesis and lipolysis [111]. Marchantin C, bibenzyl compound extracted from liverworts, show the inhibition effect on α-glucosidase, and imply that it has the potential effect on obesity and diabetes [112]. Riccardin C, a bibenzyl derivative extracted from siberian cowslip is counted for natural anti-obesity product and cardioprotective agents due to enhancing of plasma HDL level and increasing the cholesterol efflux in mice [113]. Besides, a recent study demonstrated that dendrofalconerol B, which is also the byproduct of bibenzyl extracted from orchid, indicates the potential therapeutic activity on obesity at which the compound not only involved in reduction of enzymes including pancreatic lipase and α-glucosidase but also inhibits the adipogenesis of 3T3-L1 adipocytes through downregulation of adipogenic genes [114]. According to previous reports, bibenzyl and its derivatives are likely to involve in disruption of adipocyte differentiation and counterbalancing of lipid metabolism of adipose tissue, which are required for prevention and treatment of obesity [108,112,113]. A plant bioactive compound, 4,5,4′-trihydroxy-3,3′-dimethoxybibenzyl (TDB) is extracted from a Thai orchid, *Dendrobium ellipsophyllum* (Fig. 11) and it has been documented for potent anti-cancer activity in human lung cancer cells via Akt signaling cascade [26, 27]. However, the anti-adipogenic effect of TDB and its related underlying mechanisms has not been evaluated.

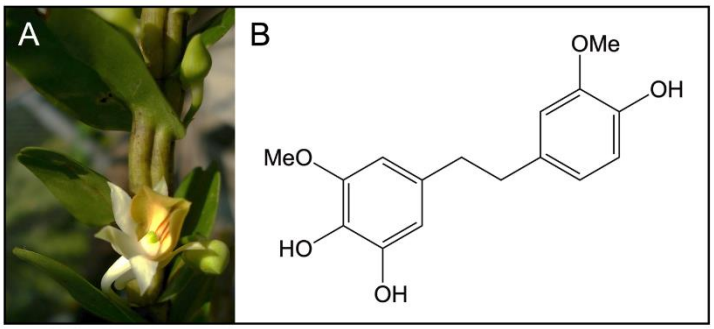


Figure 11. 4,5,4′-Trihydroxy-3,3′-dimethoxybibenzyl (TDB) derived from *Dendrobium ellipsophyllum*. An orchid species, *D. ellipsophyllum* (A) was used as a source for TDB.

Research Questions

1. Does 4,5,4′-trihydroxy-3,3′-dimethoxybibenzyl (TDB) extracted from *Dendrobium ellipsophyllum* inhibit the adipogenesis in murine 3T3-L1 preadipocytes?
2. What are the possible underlying mechanisms involved in the anti-adipogenic effect of 4,5,4′-trihydroxy-3,3′-dimethoxybibenzyl (TDB) extracted from *Dendrobium ellipsophyllum* in murine 3T3-L1 preadipocytes?

Objectives

1. To evaluate the anti-adipogenic effect of 4,5,4′-trihydroxy-3,3′-dimethoxybibenzyl (TDB) extracted from *Dendrobium ellipsophyllum* in murine 3T3-L1 preadipocytes
2. To investigate the related underlying mechanisms of 4,5,4′-trihydroxy-3,3′-dimethoxybibenzyl (TDB) extracted from *Dendrobium ellipsophyllum* during adipocyte development in murine 3T3-L1 preadipocytes

Hypothesis

4,5,4′-trihydroxy-3,3′-dimethoxybibenzyl (TDB) extracted from *Dendrobium ellipsophyllum* can exhibit the anti-adipogenic effect in murine 3T3-L1 preadipocytes via suppression not only MCE-related proteins but also Akt signaling pathway and AMPK mediated pathway consequence with downregulation of adipogenic transcription markers and lipogenic markers.

Expected benefits

The findings from this study will facilitate the development of bibenzyl extracted from *Dendrobium ellipsophyllum* as a safe and effective compound for prophylactic and therapeutic purposes on obesity.

CHAPTER III

METHODOLOGY

3.1. Materials

3.1.1. Chemicals reagents

TDB with the purity of 98% w/w were isolated from *D. ellipsophyllum* as previously described. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hoechst33342, propidium iodide (PI), oil red O, isobutylmethylxanthine (IBMX), dexamethasone, dimethyl sulfoxide (DMSO) and skim milk powder were supplied from Sigma Sigma-Aldrich (St. Louis, MO, USA). Insulin was bought from Himedia (Mumbai, India). Antibodies against LPL and SREBP1 were acquired from Invitrogen (Waltham, MA, USA) and other antibodies specific to Cyclin D1, Cyclin D3, CDK2, CDK4, CDK6, p18 INK4C, p21 Waf1/Cip1, p27 Kip1, PPARγ, C/EBPα, FAS, FABP4, PLIN1, Adiponectin, p-AMPKα (Thr172), AMPKα, p-AMPKβ1 (Ser182), AMPKβ1/2, p-ACC (Ser79), ACC, p-Akt (Thr308), Akt, p-GSK3β (Ser9), GSK3β, β-actin, and horseradish peroxidase (HRP)-coupled anti-rabbit or anti-mouse secondary antibodies were procured from Cell Signaling Technology (Danvers, MA, USA). Immobilon Western chemiluminescent HRP substrate was obtained from Millipore (Billerica, MA, USA). Chemiluminescent ImageQuant LAS 4000 was purchased from GE Healthcare Bio-Sciences AB (Björkgatan, Uppsala, Sweden).

3.2. Methods

3.2.1. Cell culture and adipocyte differentiation

Mouse embryonic preadipocyte 3T3-L1 cells (ATCC, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 units/mL of penicillin/streptomycin and 2 mmol/L of L-glutamine (Gibco, Gaithersburg, MA, USA). To induce into the adipocytes, preadipocyte differentiation was done after 2 days of confluency (Day 0) with cocktail

containing culture media supplemented with 0.5 mM IBMX, 1 µM dexamethasone and 5 µg/mL insulin for 2 days. Afterwards, the differentiation media was replaced with maintenance media containing insulin. On Day 4, the cells were restored in culture media with the change of media every 2 days until the appearance of lipid droplets are perceived under microscope [114].

3.2.2. Cell viability assay

To access the effect of TDB on the viability of preadipocytes and adipocytes, MTT viability assay were performed as previously described [115] with slight modifications. The preadipocyte cells were seeded into 96-well plates at density of 2×10^3 cells/well and allowed to attach overnight at 37ºC. Then, the cells were further treated with TDB (0-50 µM) for 48 h before adding 100 µL of MTT (0.45 mg/mL) solution to evaluate the preadipocytes viability. To determine the TDB effect on adipocytes, the preadipocytes were cultured in the 24-well plates at the density of 2×10^4 cells/well and then induced to differentiated cells according to the method described in Section (2.1). On Day 8, the culture media was replaced with media containing TDB (0-10 µM) and then incubated for 48 h prior to addition of MTT solution. After incubation with MTT solution for 3 h in the dark, the optical density (OD) of purple formazan product dissolved in DMSO was measured at 570 nm by using a microplate reader (Anthros, Durham, NC, USA). The cell viability percentage was presented in the relative OD ratio between treated to non-treated cells.

3.2.3. Nuclear staining assay

The cytotoxicity effect of TDB on preadipocytes and adipocytes were further confirmed using the co-staining of Hoechst33342 and propidium iodide (PI). After treated with indicated concentrations of TDB for 48 h, the cells were incubated with nuclear staining solution containing 2 µg/mL of Hoechst33342 and 1 µg/mL of PI

for 30 min. Mode of cell death was examined under a fluorescence microscope (Olympus IX51 with DP70, Olympus Corp., Shinjuku-ku, Tokyo, Japan) [116].

3.2.4. Determination of accumulated lipids by Oil Red O staining

Intracellular lipid content presenting in differentiated adipocytes was visualized using Oil Red O staining. After adipocyte differentiation, the cells were fixed with 10% formalin for 45 min and further incubated with Oil Red O staining solution at the room temperature for 30 min. The representative images of lipid droplets were collected by Nikon Ts2 inverted optical microscope (Tokyo, Japan). Lastly, the retained dye was extracted for quantitative analysis using absolute isopropanol to examine the OD at 510 nm by microplate reader (Anthros, Durham, NC, USA). The OD of Oil Red O was normalized to the total protein content which is determined by bicinchoninic acid (BCA) protein assay kit (Thermo-Fisher, Rockford, Illinois, USA) and presented as relative value to non-treated control cells [117].

3.2.5. Quantification of intracellular triglyceride

To explore the TDB effect on cellular triglyceride content, mutual adipocytes were washed with ice-cold phosphate-buffered saline (PBS). Then, the cellular lipid was eluted and purified by the method of Folch using chloroform/methanol, 2:1 (v/v). Finally, cellular triglyceride contents were quantified using a commercial triglyceride quantification colorimetric kit (Sigma-Aldrich, St. Louis, MO, USA) according to manufacturer's instructions at 570 nm using microplate reader (Anthros, Durham, NC, USA) [118, 119].

3.2.6. Quantification of glycerol release content

Modulating effect of TDB on lipid metabolism in mutual adipocytes was measured via estimation of released glycerol in the culture media. Fully differentiated adipocytes were treated with indicated concentrations of TDB (0-10

μM) for 48 h, and thereafter, the amount of free glycerol release was analyzed as directed by the protocol of commercially available glycerol assay kit (Sigma-Aldrich, St. Louis, MO, USA). Briefly, 10 μL of the medium from each sample were transferred into a new 96-well plate and 100 μL of the free glycerol assay reagent was introduced to each well. After 20 min of incubation in the dark place at room temperature, the OD at 570 nm was determined using microplate reader (Anthros, Durham, NC, USA) [107].

3.2.7. Cell proliferation assay

Crystal violet staining was performed to evaluate the effect of TDB on cellular proliferation. Preadipocytes were induced to differentiated and incubated in the presence or absence of TDB (0.5-5 μM) for 24-72 h. At the specific time point, the dead cells were washed twice with de-ionized water (100 μL) before fixed with 1% (w/v) formaldehyde for 30 min and further incubated with 0.05% (w/v) crystal violet for 30 min. Excess dye was washed twice with 100 μL de-ionized water and the wells were left air-dried overnight. The crystal violet-stained cells were solvated in methanol and the OD was analyzed by a microplate reader (PerkinElmer Inc, Waltham, MA, USA) at 570 nm. Percent inhibition of proliferation was evaluated in the relative OD ratio of treatments to untreated controls at same time points [120].

3.2.8. Flow cytometric analysis

Flow cytometry analysis was carried out to access the percentage of cells in different phases of cell cycle. After induction of differentiation with or without TDB (0.5-5 μM) for 24 h, the adherent cells were trypsinized and collected by centrifugation. Then, the cells were fixed with 70% ethanol (400 μL) for 2 h on ice. After rinsing with PBS for two times, the fixed cells were again incubated in a staining solution containing RNase A (60 μg/ml) and propidium iodine (PI) (50 μg/ml) for 1 h at 37°C. The cells were analyzed using Guava easy Cyte flow cytometer programmed

with InCyte 3.3 software (EMD Millipore, Austin, US) and the density of cells in G0/G1, S and G2/M phase were measured with FlowJo V10 software trail version (Williamson Way, Ashland, US) [120].

3.2.9. Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) analysis

Cells were treated with TDB (0-5 μM) along with differentiation or maturation media. RNA samples were extracted from preadipocytes and adipocytes with or without sample to evaluate the mRNA expression at the differentiation level and at the mutual adipocytes through collection on day 4 and day 10, respectively. Total RNA was reverse transcribed into cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Rockford, IL, USA). cDNA was then quantified by measuring the absorbance at 260 nm using Thermo Scientific NanoDrop One microvolume UV-Vis spectrophotometers (Thermo Scientific, Rockford, IL, USA). The RT-qPCR was carried out by Luna Universal qPCR Master Mix (Bio-Rad Laboratories, Hercules, CA, USA). Ultimately, the gene expression levels were quantified in Cq value using reverse transcription quantitative real-time PCR (RT-qPCR) along with the CFX 96 Real-Time PCR system (Bio-Rad Laboratories, Hercules, CA, USA) under the following conditions, which include the initial denaturation step at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 5 sec and primer annealing at 55°C for 30 sec. The relative mRNA expression levels of targeted genes were normalized to β-actin using the $2^{-\Delta\Delta CT}$ method [27].The designed primer sequences used in this amplification study were indicated in Table 2.

Table 2. Primer sequences of related-adipogenesis mechanism

Gene	Forward (5' - 3')	Reverse (5' - 3')
PPARγ	GATTCTCCTRTTGACCCAG	GARTGSGAGTGGTCTTCCAT
C/EBPα	AGTCGGTGGACAAGAACAGC	GTGTCCAGTTCRCGGCTCA
SREBP1	YTGCMGACCCTGGTGAGTG	GASCGGTAGCGCTTCTCAAT
FAS	AAYGCCGGCACCAATACAGA	ATGCCATTCAGYTCCTGG
FABP4	AARTCACYGCAGAYGACAG	AACTCTYGTGGAAGTSACG
LPL	AGAGAGAGGACTYGGAGAYG	CAACTCARGCAGAGCCCTTT
PLIN1	CACCATCTCYACCCGCCTYC	GGWACTCYACCACCTTCTC
Adiponectin	YCCCAAYRTRCCCATTCGCTT	GMAYRGCCTTGTCCTTCTTG
β-Actin	CCACCATGTACCCWGGCATT	CGGACTCRTCRTACTCCTGC

PPARγ, peroxisome proliferator-activated receptorγ; C/EBPα, CCAAT/enhancer-binding proteinα; SREBP1, sterol regulatory element binding protein-1; FAS, fatty acid synthase; FABP4, fatty acid binding protein 4; LPL, lipoprotein lipase; PLIN1, Perilipin 1.

3.2.10. Western blot analysis

After indicated treatment in differentiation or maturation media, 3T3-L1 cells were washed with phosphate buffered saline (PBS, pH 7.4). Next, radio-immunoprecipitation assay (RIPA) buffer (Thermo scientific, Rockford, Illinois, USA) supplemented with protease inhibitor cocktail (Roche Applied Science, Indianapolis, Indiana, USA) was added and incubated on ice to break the cell membrane for 45 min. The cell lysates were centrifuged at 12,000 rpm at 4ºC for 15 min to collect the clear supernatant containing cellular protein at which BCA protein assay kit was used to measure the total protein content. Total protein (30 µg) from each sample was

loaded and separated onto 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel electrophoresis. Then, the separated proteins were transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA) which were blocked with 5% skim milk in TBST buffer (Tris Buffered Saline with 0.1% Tween 20, pH 7.2). The membranes were further immunoblotted with targeted primary antibodies at 4ºC for overnight. The membranes were washed with TBST for three times (7 min) before probing in related horseradish peroxidase (HRP)-linked secondary antibodies at room temperature for 2 h. Finally, the reactive protein signals exposed with chemiluminescent substrates were presented under UV and quantified using Chemiluminescent ImageQuant LAS 4000 (GE Healthcare Bio-Sciences AB, Björkgatan, Uppsala, Sweden) [27].

3.2.11. Statistical analysis

Numerical results were reported as means ± standard deviations (SDs) derived from at least three independent experiments (n = 3). Statistical comparison of means by student t-test or one-way analysis of variant (ANOVA) with Tukey's post hoc test was performed at a significance level (α) of 0.05 using GraphPad Prism 8.0.2 software (San Diego, CA, USA). The significance *p*-value derived from each statistical comparison was addressed.

3.3. Conceptual framework

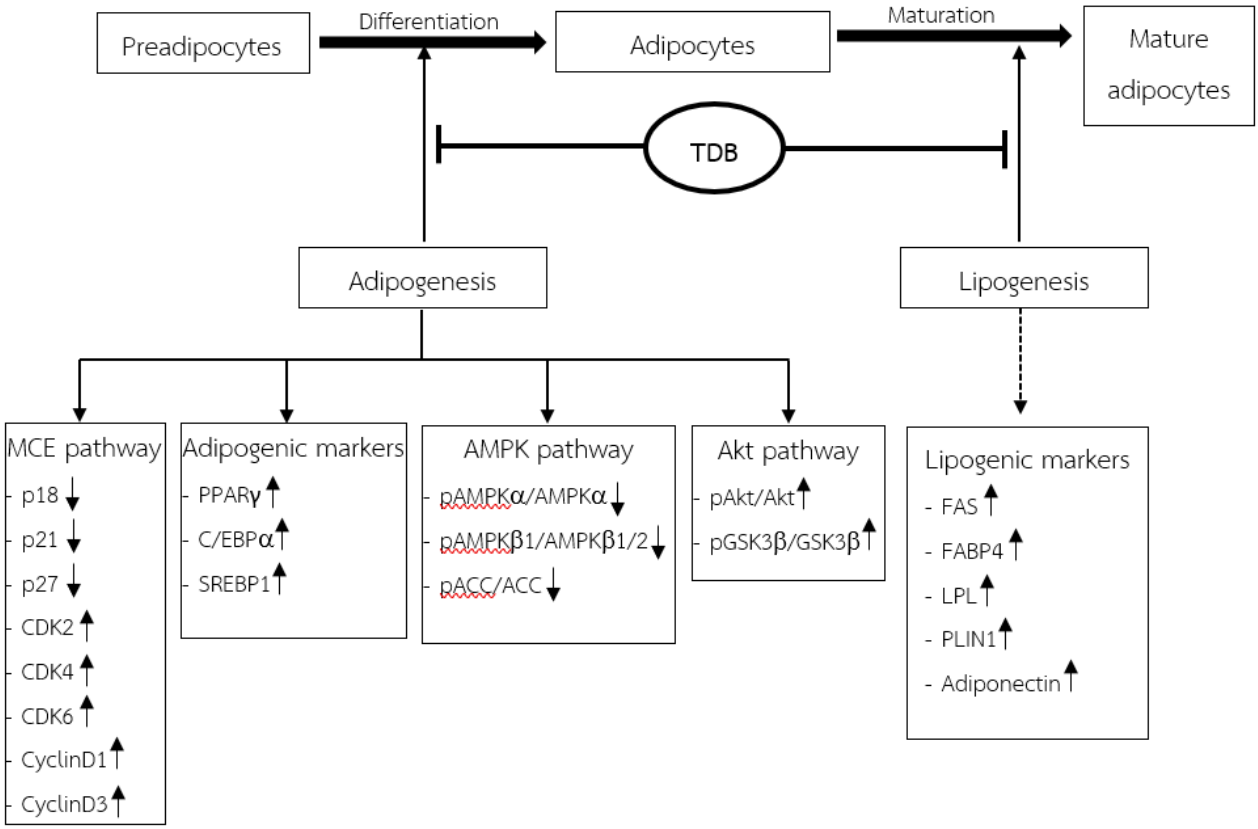


Figure 12. Conceptual framework

3.4. Experimental design

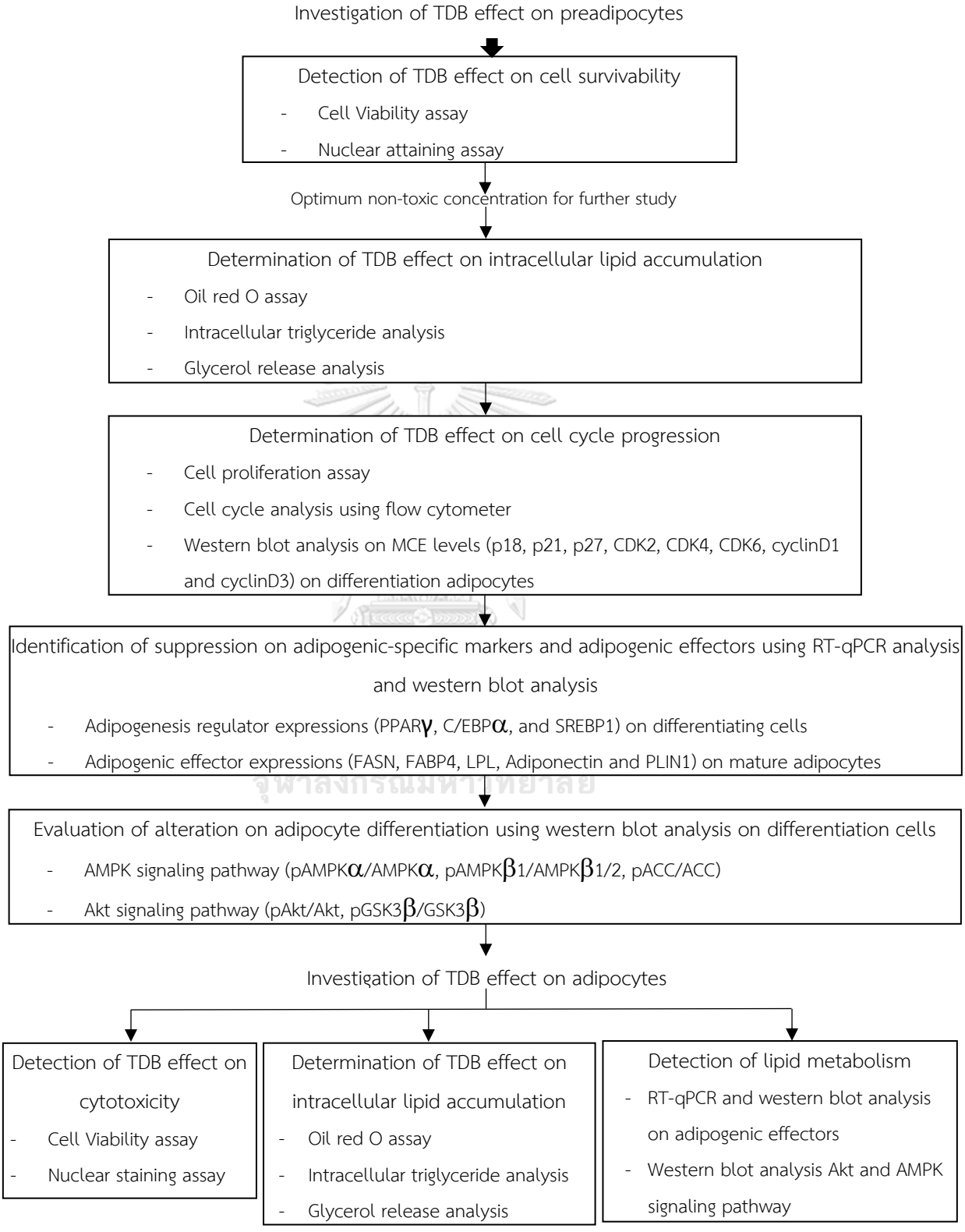


Figure 13. Experimental design

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1. Cytotoxic profiles of TDB

Cytotoxic effects of TDB on murine (3T3-L1) and human (PCS-210-010) preadipocytes and adipocytes were likely to be varied in a dose-dependent manner (Fig. 14). Cell viability percentages for preadipocytes significantly decreased after treatments with ≥10 μM TDB (Fig. 14A and C), while milder cytotoxic effects (≥25 μM TDB) were observed for adipocytes (Fig. 14E and G). These results were supported by significantly higher half maximal inhibitory concentration (IC50) values for adipocytes than those assessed with preadipocytes ($p < 0.001$). The shift in cytotoxicity indicates the susceptibility of cells after getting exposed to a tested compound. It was explicit that preadipocytes were more susceptible to TDB than those differentiated. This variation in cellular responses might be due to the alteration in cell physiology, complexity, and robustness caused by differentiation [114, 115]. These data should be considered to optimize drug dosing in the future. There was no significant difference in IC50 values of TDB between murine and human cell lineages (Fig. 14). This cross-species similarity of TDB effects might result from the conservation of adipogenic genes that regulate terminal differentiation across multiple species, including mouse and human lineages [116]. Based on these findings, non-cytotoxic concentrations of TDB were defined into a range of 0.5 – 5 μM. Moreover, cells exposed to these doses did not show apoptosis after nuclear staining assays (Fig. 14B, D, F, and H), which we could exclude cytotoxic-induced cell death caused by TDB from its principal effects on adipocyte differentiation.

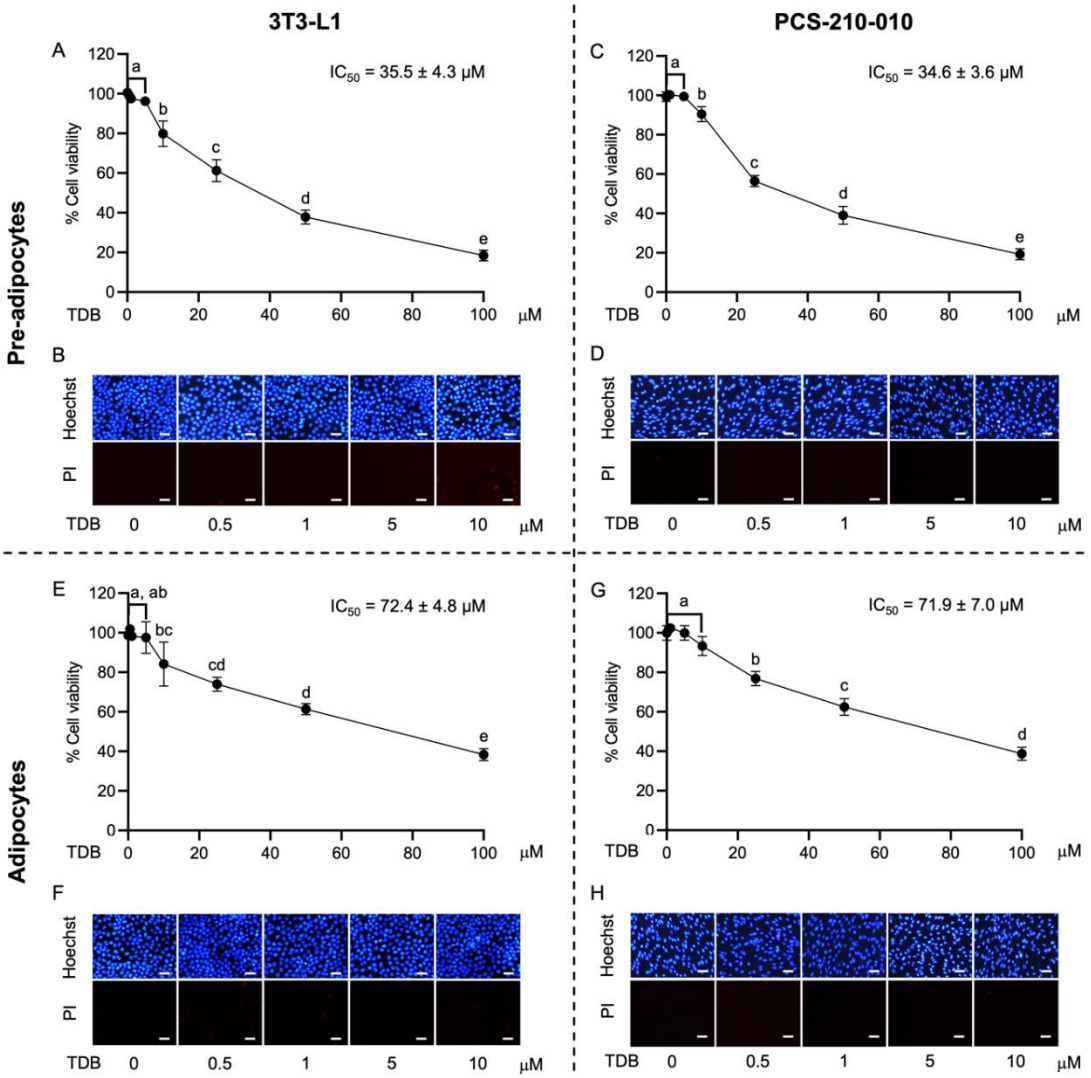


Figure 14. Cytotoxic effects of 4,5,4′-trihydroxy-3,3′-dimethoxybibenzyl (TDB) on preadipocytes and adipocytes. The percentage of cell viability (a) and nuclear staining (b) with Hoechst33342 (Hoechst) and propidium iodide (PI) were conducted after testing 3T3-L1 and PCS-210-010 preadipocytes and adipocytes with TDB for 48 h. Graphical results demonstrating means ± SDs and the half maximal inhibitory concentration (IC50) values were created using GraphPad Prism. Different small case letters in each graph refer to the significant difference between means compared by one-way ANOVA with Tukey's post hoc test at α = 0.05, n = 3. Scale bars = 50 μm.

4.2. Roles of TDB in cellular lipid metabolisms

As adipocytes have their vital roles in lipid metabolisms to regulate energy homeostasis, lipogenesis and lipolysis are central activities often investigated to validate the intrinsic functions of these cells [117-119]. Such major activities of 3T3-L1 and PCS-210-010 cells after treating with non-cytotoxic doses of TDB at early and late differentiation were tracked and assessed with Oil Red O staining and the contents of cellular triglyceride and glycerol released (Fig. 15). Cellular lipid accumulation significantly relied on differentiation inducers ($p < 0.001$), supported by the absence of cellular lipid droplets stained with Oil Red O (Fig. 15A, C, E, and G) and nearly zero percentage of Oil Red O stained (Fig. 15B, D, F, and H) in TDB-free undifferentiated controls. These results confirmed that differentiation inducers were essential to evolve mature adipocytes from preadipocytes as well as their functions in cellular lipid metabolisms, which was consistent with many other studies [90, 97]. When considering TDB-free differentiated controls of both cell lineages, the contents of cellular triglyceride and glycerol released during maturation were significantly higher than those measured at early differentiation ($p < 0.001$) (Fig. 15B, D, F, and H). Such increases at late differentiation would result from the progress in adipocyte development that gradually enhanced the cellular capability to function in lipid metabolisms. Numerous studies proved that several genes involved in cellular lipogenesis and lipolysis were increasingly expressed from early to late adipocyte differentiation [22, 23].

The Oil Red O-stained percentage and cellular triglyceride content of both cell lineages significantly decreased after treating cells with increased TDB doses, which reversely correlated to glycerol content released from cells (Fig. 15B, D, F, and H). As non-cytotoxic concentrations of TDB were used in this work, the viability and susceptibility of cells should not be the causes of such diminished lipogenesis and

enhanced lipolysis. Adipocyte differentiation modulated by TDB would solely be responsible for these changes. Similar effects of TDB in a dose-dependent manner were observed in some previous studies using 3T3-L1 cells tested with oxyresveratrol, cyanomaclurin [111], and dendrofalconerol B [107]. The degrees of TDB effects on cellular lipid metabolisms were higher when cells were exposed to the compound at early differentiation because TDB at 0.5 μM did not show significant differences compared to TDB-free differentiated controls during adipocyte maturation (Fig. 15B, D, F, and H). In addition to the increased robustness of mature adipocytes compared to preadipocytes, introducing TDB at different time points that held altered levels of inducers, regulators, and effectors in adipocyte differentiation would be another reason for such decreased effects of TDB during maturation. A study proposed that a tested compound might have inhibitory effects on some inducers required to initiate adipocyte differentiation [107]. Another criterion to consider is the cellular uptake rate when preadipocytes and adipocytes with higher lipid deposits are exposed to TDB, which relies on the cellular strength and the tested compound's physicochemical properties. This hypothesis is still unproven but needs further investigation to optimize drug dosing and delivery. Based on the cytotoxic effects and roles in cellular lipid metabolisms of TDB observed with 3T3-L1 and PCS-210-010 cells were not significantly different, we chose 3T3-L1 cells as the models to elucidate the underlining molecular mechanisms of TDB in adipocyte differentiation.

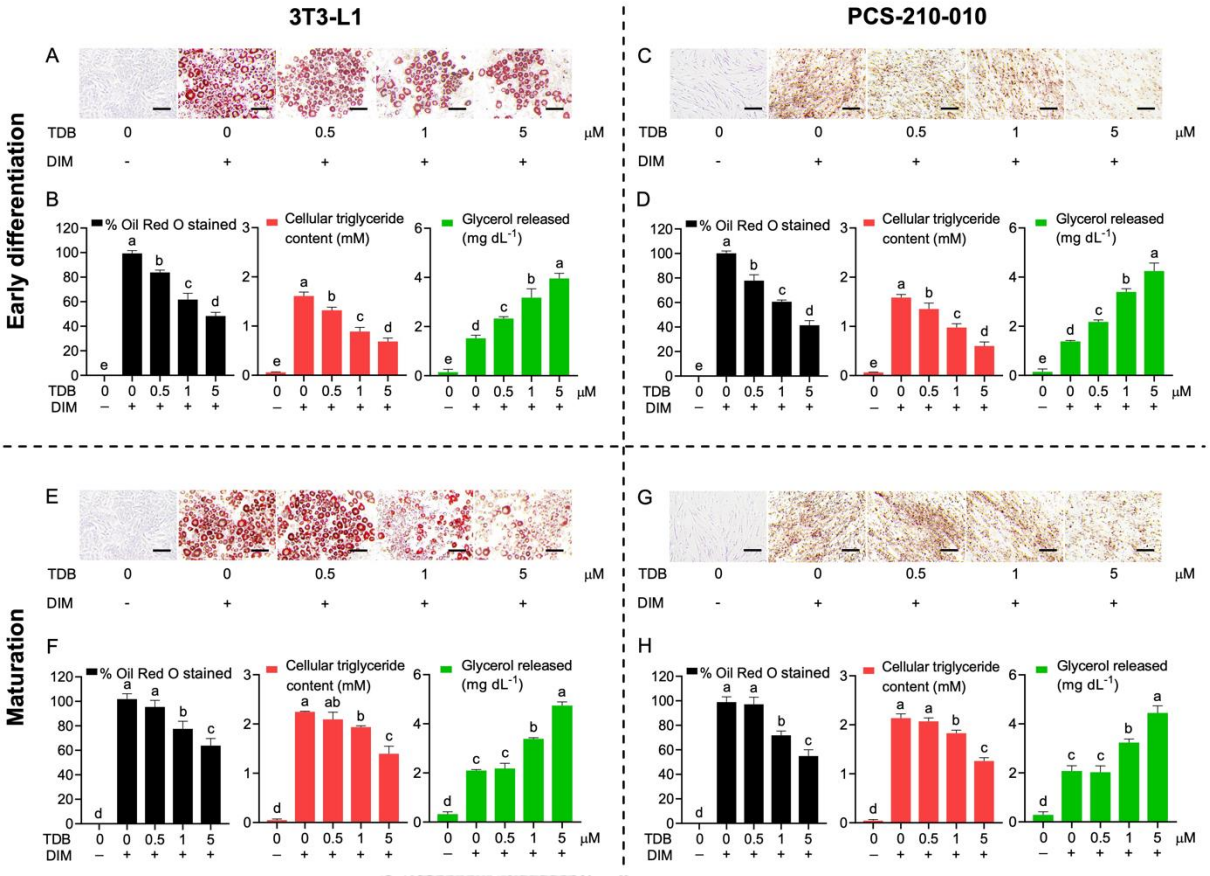


Figure 15. Impacts of 4,5,4′-trihydroxy-3,3′-dimethoxybibenzyl (TDB) on lipid metabolisms of 3T3-L1 and PCS-210-010 cells. Images demonstrate Oil Red O staining; scale bars = 50 μm (a). The percentage of Oil Red O stained, the cellular triglyceride content, and the glycerol released were assessed (b). Any tests were conducted in the absence (-) or presence (+) of differentiation media (DIM) at early differentiation and during maturation. Graphical results demonstrating means ± SDs were created using GraphPad Prism. Different small case letters in each graph refer to the significant difference between means compared by one-way ANOVA with Tukey's post hoc at α = 0.05, n = 3.

4.3. Suppression effect of TDB on mitotic clonal expansion through halting cell cycle progression

TDB could suppress cell proliferation and maintain growth arrest preadipocytes at early differentiation (Fig. 16). These growth arrest preadipocytes typically undergo a few rounds of mitosis to increase the number of cells before entering adipocyte differentiation [10-12]. In the absence of differentiation inducers, most proliferated quiescent preadipocytes remained at the G0/G1 phase in the cell cycle due to elevated levels of cell cycle suppressors like CDKIs (e.g., p18, p21, and p27) [70]. Our findings proved that cell proliferation and cell cycle progression were significantly triggered by the established adipocyte differentiation program (Fig. 16). After treating cells with TDB at non-cytotoxic doses, the compound significantly decreased percentages of 3T3-L1 cell proliferation (Fig. 16A). Interestingly, cell proliferation measured at 48 and 72 h after testing with TDB at 5 μM were in the equilibrium compared to TDB-free differentiated controls of the 24 h assay (Fig. 16A). This result was confirmed by flow cytometry-based cell cycle progression percentages (Fig. 16B and C), in which 5 μM TDB-treated cell population counted at the G0/G1 phase was not significantly different from that detected in TDB-free undifferentiated controls (Fig. 16C).

Another support with protein expression studies unveiled that TDB significantly downregulated CDKs and cyclins D1 and D3 but significantly upregulated CDKIs (Fig. 17). It is conceivable that CDK4 and CDK6 play a principal role in the cell cycle progression during the G0/G1 phase, while CDK2 functions during the G1/S interphase [18]. Previous studies reported that decreased CDKs and increased CDKIs were responsible for disrupting MCE and adipocyte differentiation. A study revealed that cyclin D1 could inhibit the function of liganded PPAR**γ** from promoting adipocyte differentiation [69]. Based on this evidence, cyclin D1 downregulation due to TDB treatment could progress adipocyte differentiation. Remarkably, such a

diminished level of cyclin D1 after treating cells with TDB at 5 μM was still significantly higher than that of TDB-free undifferentiated control (Fig. 17B). Hence, this level of cyclin D1 would be sufficient to restrain cell proliferation and cell cycle progression, which consequently repressed adipocyte differentiation. For the expression of cyclin D3 after treating cells with TDB at 5 μM, it did not significantly differ from that observed in TDB-free undifferentiated control (Fig. 17C). The decreased cyclin D3 was proved to be a reason for suppressing adipocyte differentiation [120].

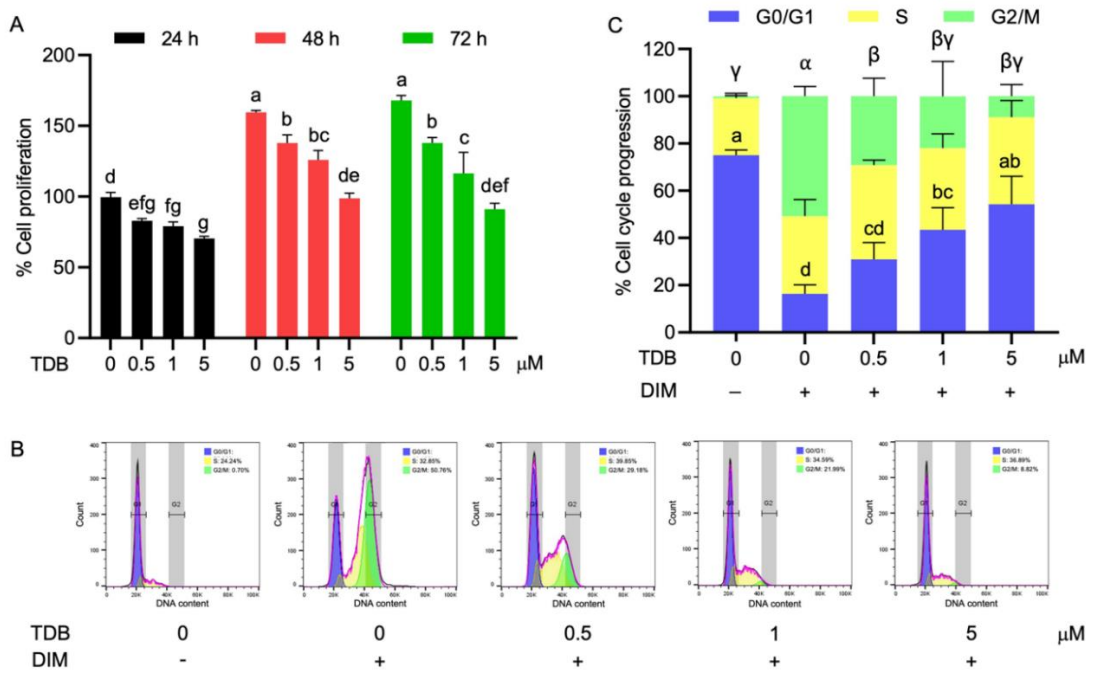


Figure 16. Cell proliferation and cell cycle progression after treating 3T3-L1 cells with 4,5,4′-trihydroxy-3,3′-dimethoxybibenzyl (TDB). Cells treated with TDB for 24, 48, and 72 h were assessed for the percentage of cell proliferation using crystal violet staining (a). Flow cytometry was conducted to classify TDB-treated cells (of 24 h treatments) into G0/G1, S, and G2/M phases in the cell cycle (b, a set of representative data). Cell population per phase in the cell cycle was accounted in the percentage (c). The tests were carried out in the absence (-) or presence (+) of

differentiation media (DIM) at early differentiation. Graphical results demonstrating means ± SDs were created using GraphPad Prism. Different small case letters or different Greek alphabets for the G2/M phase refer to the significant difference between means compared by one-way ANOVA with Tukey's post hoc at α = 0.05, n = 3.

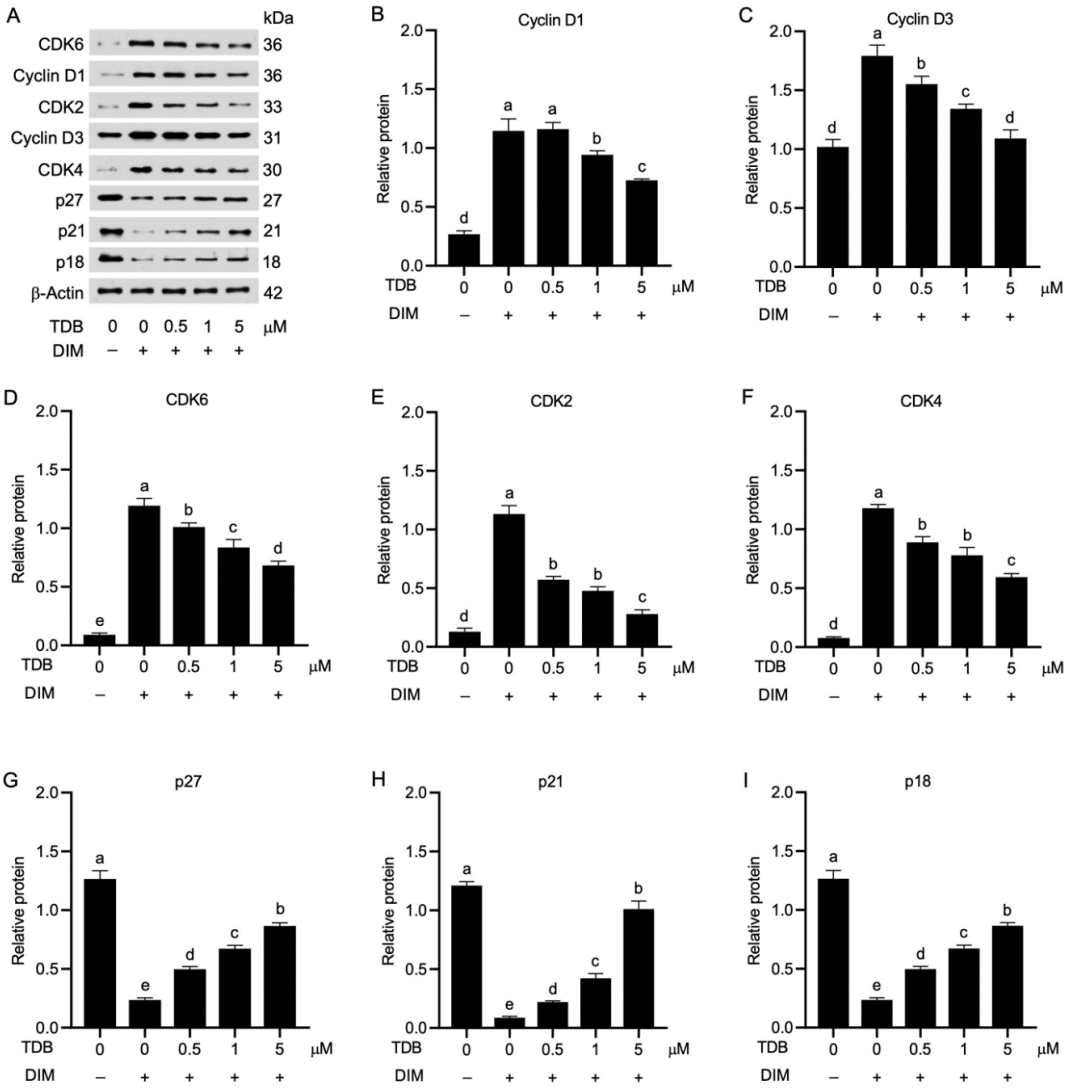


Figure 17. Expressions of some mitotic clonal expansion (MCE)-related proteins after treating 3T3-L1 cells with 4,5,4′-trihydroxy-3,3′-dimethoxybibenzyl (TDB). The band intensity of each protein obtained from western blot analysis (a) was employed to estimate the relative protein level using β-Actin as a reference (b-i). MCE-related

proteins include cyclin D1, cyclin D3, cyclin-dependent kinase (CDK) 6, CDK2, CDK4, and cyclin-dependent kinase inhibitors (i.e., p27, p21, and p18). The tests were carried out in the absence (-) or presence (+) of differentiation media (DIM) at early differentiation. Graphical results demonstrating means ± SDs were created using GraphPad Prism. Different small case letters in each graph refer to the significant difference between means compared by one-way ANOVA with Tukey's post hoc at α = 0.05, n = 3.

4.4. Inhibitory effect of TDB on adipogenesis

Notably, the crosstalk between MCE-related proteins and adipogenic regulators is necessary for initiating adipocyte differentiation [10]. Adipogenic regulators inhibit MCE and are crucial in the progress of adipocyte development, cellular lipid metabolisms, and obesity incidence [22, 19]. Downregulations of adipogenic regulators, PPARγ, C/EBPα, and SREBP1 at early adipocyte differentiation caused by TDB were consistent with their encoding genes, *Pparg*, *C/ebpa*, and *Srebp1* (Fig. 18). The impacts of TDB on the suppressions of these regulators significantly varied in a dose-dependent manner (Fig. 18B-E). Considering TDB-free differentiated controls, PPARγ and C/EBPα were substantially more abundant than SREBP1 (Fig. 18B-D). A previous study reported that *Pparg*-knocked out mice had impaired lipid metabolisms and were resistant to high-fat diet (HFD)-induced obesity [121]. Likewise, suppressed adipocyte differentiation and impaired cellular lipid metabolisms were observed in *C/ebpa*-deleted 3T3-L1 cells and mice [80]. Another evidence confirmed that the overexpression of *Srebp1* in mouse adipose tissues accelerated fat formation and dysregulated fatty acid homeostasis [82]. Thus, suppressing the expressions and functions of these adipogenic regulators at early adipocyte differentiation by TDB would offer a promising way to prevent obesity incidence.

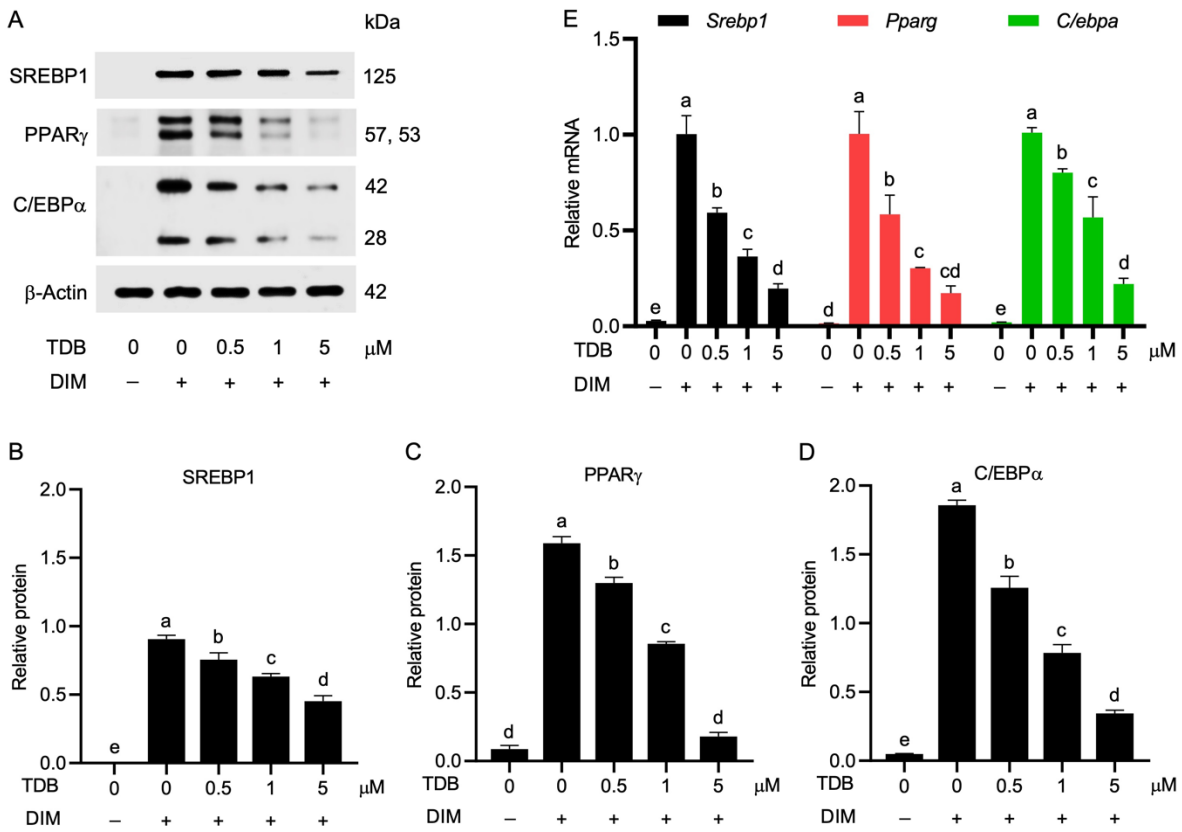


Figure 18. Expressions of some adipogenic regulators after treating 3T3-L1 cells with 4,5,4′-trihydroxy-3,3′-dimethoxybibenzyl (TDB). The band intensity of each protein obtained from western blot analysis (a) was employed to estimate the relative protein level using β-Actin as a reference (b-d). Adipogenic regulators include sterol regulatory element-binding protein-1 (SREBP1), peroxisome proliferator-activated receptor γ (PPARγ), and CCAAT enhancer-binding protein α (C/EBPα). Expressions of genes encoding these adipogenic regulators (i.e., Srebp1, Pparg, and C/ebpa) were confirmed using RT-qPCR (e). The tests were conducted in the absence (-) or presence (+) of differentiation media (DIM) at early differentiation. Graphical results demonstrating means ± SDs were created using GraphPad Prism. Different small case letters for each protein or each gene refer to the significant difference between means compared by one-way ANOVA with Tukey's post hoc at α = 0.05, n = 3.

4.5. Inhibitory effect of TDB on lipogenesis

Another role of adipogenic regulators is to stimulate the transcription of adipogenic effectors that function in cellular lipid metabolisms and determine adipocyte phenotypes. Dysregulations of adipogenic effectors-encoding genes can pose impaired adipocyte differentiation and obesity development [22]. Similar downregulation effects of TDB on adipogenic regulators were also observed with adipogenic effectors (i.e., FAS, PLIN1, LPL, adiponectin, and FABP4) and their encoding genes (i.e., *Fasn*, *Plin1*, *Lpl*, *Adipoq*, and *Fabp4*) detected at early differentiation and during maturation (Fig.19). Deletions of *Fasn*, *Plin1*, *Lpl*, *Adipoq*, or *Fabp4* in mice resulted in decreased levels of polyunsaturated fatty acids in adipose tissues and intracellular lipids uptake, restricted cellular lipid accumulation, enhanced cellular lipolysis, and low risk of postprandial hyperlipidemia, obesity-related glucose intolerance, insulin resistance, HFD-induced obesity, and atherosclerosis [78, 97-101]. Except for FAS and LPL (Fig. 19B, D, I, and K), other adipogenic effectors in this study were more abundant during adipocyte maturation than those measured at early differentiation. This trend of results agreed with what was observed by Oil Red O staining and measuring contents of cellular triglyceride and glycerol released (Fig. 15). The expressions of adipogenic effectors rely significantly on the cellular lipid contents that why it is reliable to be higher during adipocyte maturation (differentiated adipocytes launch their functions in lipid homeostasis) than those taken place at early differentiation [22].

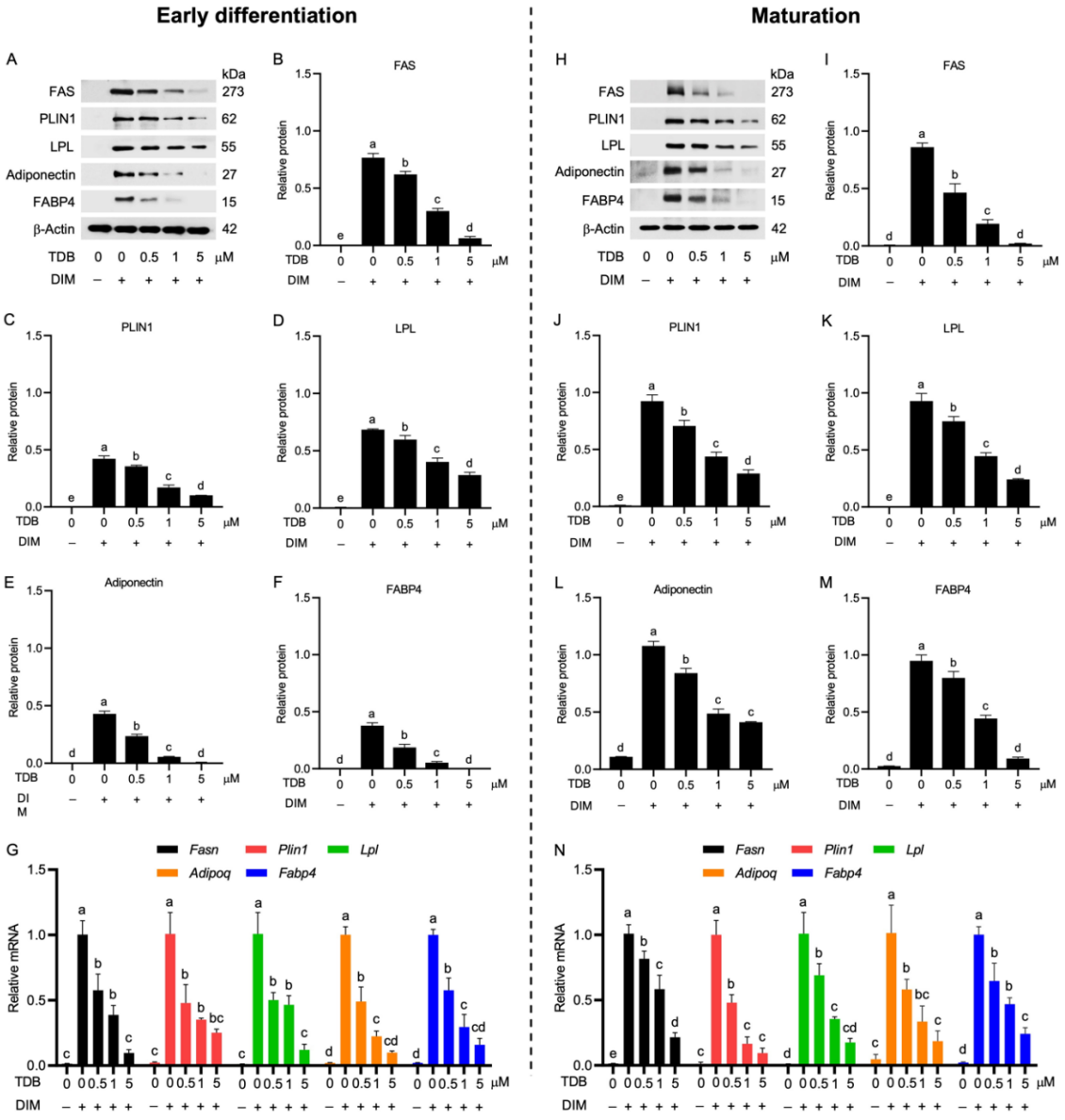


Figure 19. Expressions of adipogenic effectors after treating 3T3-L1 cells with 4,5,4′-trihydroxy-3,3′-dimethoxybibenzyl (TDB). The band intensity of each protein obtained from western blot analysis (a) was employed to estimate the relative protein level using β-Actin as a reference (b-f). Adipogenic effectors include fatty acid synthase (FAS), perilipin 1 (PLIN1), lipoprotein lipase (LPL), adiponectin, and fatty acid-binding protein 4 (FABP4). Expressions of genes encoding these adipogenic effectors (i.e., Fas, Plin1, Lpl, Adipoq, and Fabp4) were confirmed using RT-qPCR (e). The tests were conducted in the absence (-) or presence (+) of differentiation media (DIM) at early

differentiation. Graphical results demonstrating means ± SDs were created using GraphPad Prism. Different small case letters for each protein or gene refer to the significant difference between means compared by one-way ANOVA with Tukey's post hoc at α = 0.05, n = 3.

4.6. Anti-adipogenic role of TDB via AMPK and Akt upstream pathways

As regulators and effectors in adipocyte differentiation can be triggered through the AKT/GSK-3β signaling pathway and the AMPK-ACC pathway, we assessed how TDB influenced the activations of proteins involved in these pathways (Fig. 20). AKT and GSK-3β (Fig. 20A, B, C, G, H, and I) in TDB-free differentiated and undifferentiated controls were significantly activated by phosphorylation either at early differentiation or during maturation, which was contrary to AMPKα, AMPKβ, and ACC (Fig. 20A, D-G, and J-L). TDB significantly suppressed the AKT/GSK-3β signaling pathway but promoted the AMPK-ACC cascade (Fig. 20). These findings supported that reverse crosstalk between these two pathways regulated adipocyte differentiation. The sequential phosphorylation of AKT and GSK-3β promotes the cell cycle progression by suppressing CDKIs in MCE and initiates adipocyte differentiation [99]. Another study unveiled that AKT gene-knocked out mice were resistant to HFD-induced obesity with decreased PPARγ and C/EBPα [92]. The competitive phosphorylation between the two pathways can lead to the inhibitions of PPARγ, C/EBPα, and SREBP1 functions and impaired cellular lipid metabolisms [87, 88, 90]. It is conceivable that the AKT/GSK-3β signaling pathway plays a role in adipocyte hypertrophy during maturation, and its activation promotes lipogenesis with increased FAS, PLIN1, and adiponectin [122]. For the AMPK-ACC pathway, its activation can downregulate adipocyte differentiation by repressing PPARγ, CEBPα, and lipid biosynthesis with decreased FAS, FABP4, and PLIN1 [87]. Indeed, AMPK acts

as the intracellular energy sensor to counterbalance the nutrient intake with energy demand, suggesting that this protein kinase has a definite link to the pathogenic development of metabolic diseases including obesity and diabetes mellitus [83]. Recent studies unveiled that knocking out AMPKα and AMPKβ subunits in mice resulted in dysregulated lipid homeostasis, leading to obesity and insulin resistance [87, 123].

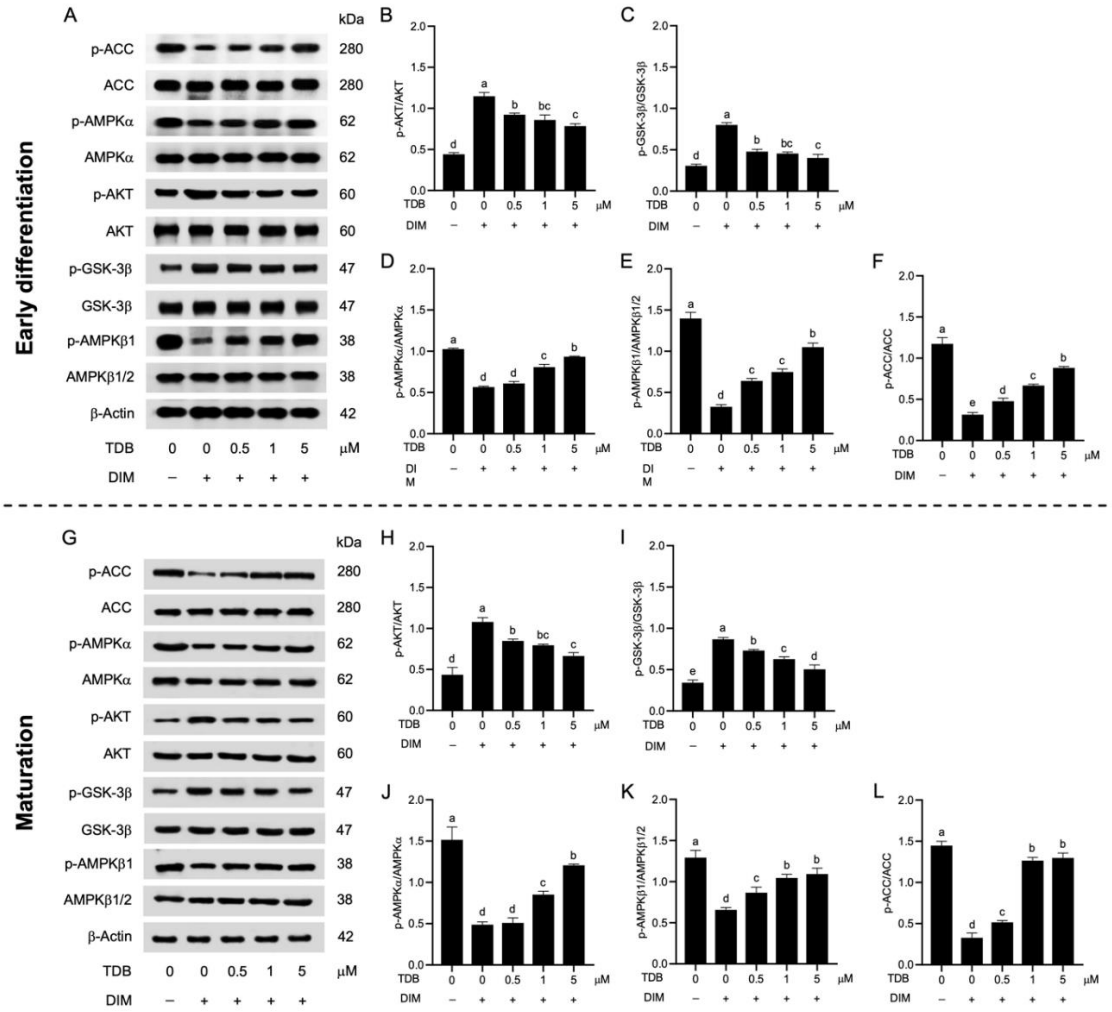


Figure 20. Phosphorylated proportions of proteins involved in some adipocyte differentiation-linked biological pathways after treating 3T3-L1 cells with 4,5,4′-trihydroxy-3,3′-dimethoxybibenzyl (TDB). The band intensity of each protein obtained from western blot analysis (a) was employed to estimate the relative protein level

using β-Actin as a reference (b-f). Analyzed proteins include protein kinase B (AKT), glycogen synthase kinase-3β (GSK-3β), 5′-adenosine monophosphate-activated protein kinases α and β1/2 (AMPKα and AMPKβ1/2), acetyl-CoA carboxylase (ACC), and phosphorylated analogs of these proteins (with p prefix). The tests were carried out in the absence (-) or presence (+) of differentiation media (DIM) at early differentiation and during maturation. Graphical results demonstrating means ± SDs were created using GraphPad Prism. Different small case letters in each graph refer to the significant difference between means compared by one-way ANOVA with Tukey's post hoc at $\alpha = 0.05$, n = 3.

Additionally, the phosphorylation cascades of Akt/GSK-3β and MEK-1/ERK 1/2 are implicated in MCE and adipocyte differentiation, and both signaling pathways have the potential to be molecular therapeutic targets for obesity [12]. According to our research data, TDB inhibited adipocyte differentiation by modulating the MCE and Akt signaling pathways. However, the involvement of TDB in the Erk signaling pathway has not yet been examined, and thus, further studies on the involvement of TDB in the Erk signaling pathway are needed to determine if anti-adipogenic activity of TDB is based on Akt and/or Erk signaling pathways.

Moreover, binding of insulin to the insulin receptor (IR) on the cellular membrane triggers the insulin receptor substrate (IRS1) to regulate the activation of Akt signaling cascades, which in turn control the processes of adipogenesis [71] and GLUT4 translocation [124]. It has been shown that TDB's anti-adipogenic activity through reduction of intracellular lipid accumulation was brought on by inactivating the Akt pathway, therefore, more research is required on the Akt-related glucose uptake mechanisms. Besides, to determine whether the TDB is a binding competitor of insulin to deactivate the Akt signaling pathway, molecular docking and competitive binding research should also be carried out.

TDB has demonstrated anti-adipogenic activity as well as inhibitory effects against human lung cancer cells and CSCs via disrupting the AKT signaling pathway [26, 27]. Akt, on the other hand, regulates a wide range of cellular processes throughout the body, including metabolism, proliferation, growth, and survival [124]. Accordingly, *in vivo* research should be carried out to assess the impact of TDB mediated Akt signaling pathway on overall body function. Furthermore, oral and topical formulation research should be conducted in order to target TDB to specific body parts using designed drug delivery systems.

CHAPTER V

CONCLUSION

Current anti-obesity drugs have food and drug administration (FDA) approval, but they do not directly influence the adipogenesis process to reduce the obesity. Moreover, their weight control and weight loss in obese patients have failed for long-term maintenance due to side effects and resistances. Adipogenesis is a novel prospective target for obesity treatment, and several bioactive natural compounds that target the differentiation and maturation stages of adipogenesis are actively studied for obesity treatment. In this regard, the present study provides insight on the potential mechanisms of TDB-mediated inhibition of adipogenesis through suppression of differentiation and maturation processes (Fig. 21). Although further research is needed to see whether TDB has the same anti-adipogenic effects *in vivo* and clinical studies, the findings of this study suggest that TDB could be a valuable dietary strategy to alleviate obesity via inhibition of adipogenesis.

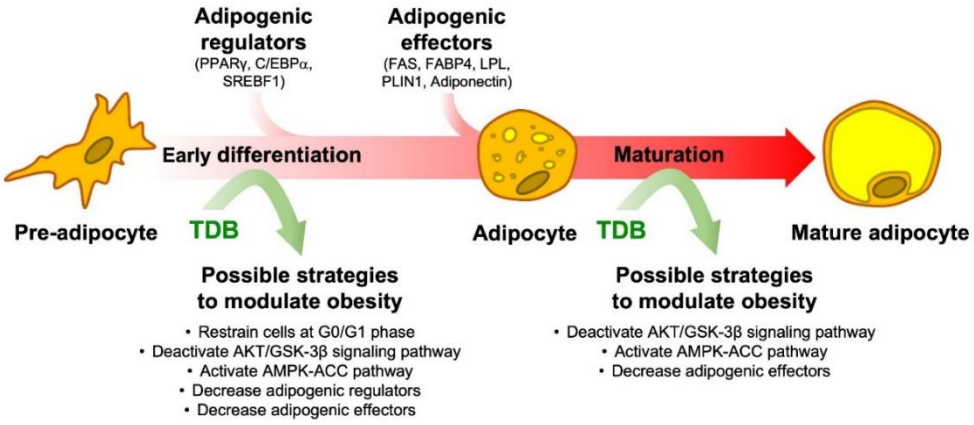


Figure 21. Graphical representation of TDB mediated anti-adipogenic activity

To this end, TDB has shown its pharmaceutical potential to regulate obesity through the modulation of adipocyte differentiation. The underlining molecular mechanisms of TDB in adipocyte differentiation include restraining the cell cycle progression at the G0/G1 phase by repressing MCE at early differentiation as

well as deactivating the AKT/GSK-3β signaling pathway and activating the AMPK-ACC cascade with decreased adipogenic regulators and effectors at early differentiation and during maturation. Overall, TDB processes the anti-adipogenic activity and could be used to develop therapeutic and preventive bioactive natural compounds for obesity, and the schematic diagram for the proposed mechanism of this anti-adipogenic activity was shown in Fig. 22.

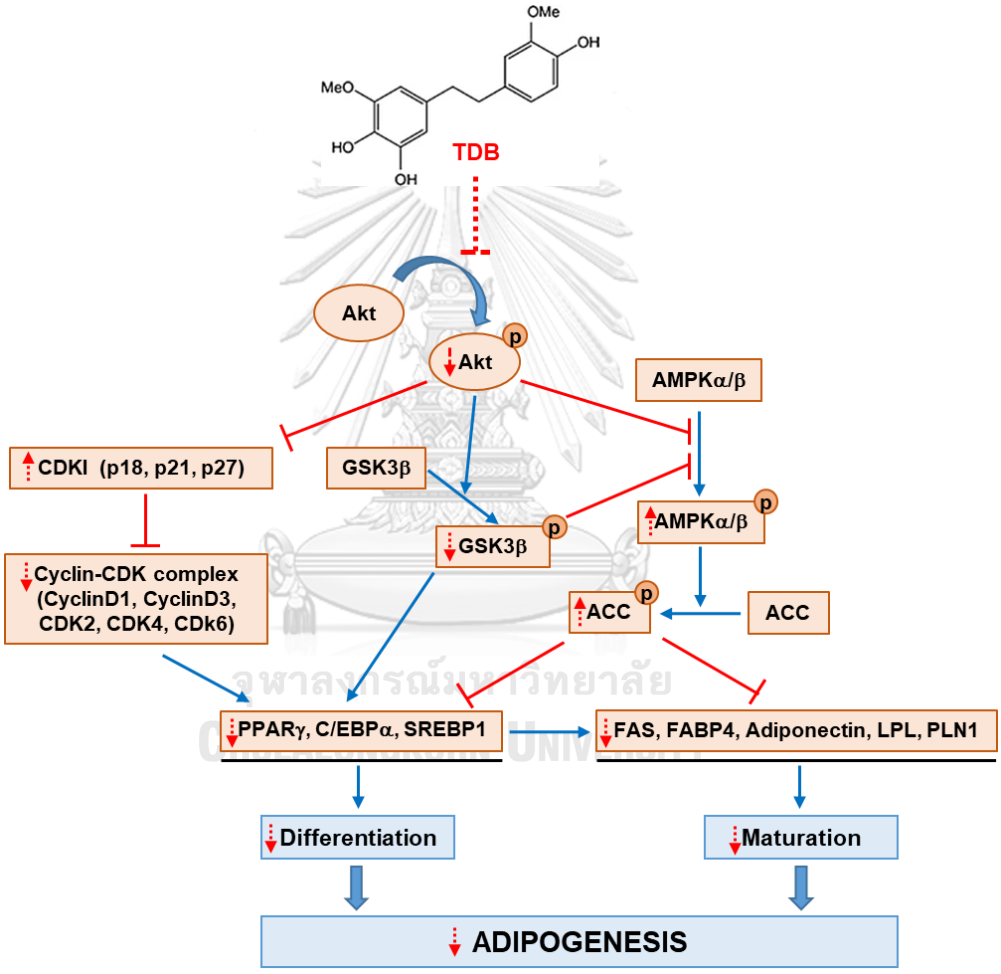


Figure 22. Proposed schematic diagram of the mechanism of anti-adipogenic effect of TDB extracted from *Dendrobium ellipsophyllum* in murine 3T3-L1 cells. TDB inhibited adipogenesis by decreasing adipogenic regulators (PPARγ, C/EBPα and SREBP1) as well as adipogenic effectors (FAS, FABP4, adiponectin, LPL, PLIN1). Moreover, TDB suppressed the MCE pathway, downregulated the Akt signaling pathway, and upregulated the AMPK signaling pathway to reduce adipogenesis.

APPENDIX

TABLES AND FIGURES OF EXPERIMENTAL RESULTS

Table 3. The percent cell viability of 3T3-L1 preadipocytes (a) and adipocytes (b) after treatment with 0 - 100 μM of TDB

(a)

TDB (μM)	Cell viability (%)
0	100.58 ± 1.43[a]
0.5	99.07 ± 1.93[a]
1	97.51 ± 1.31[a]
5	96.26 ± 1.03[a]
10	79.84 ± 6.42[b]
25	61.24 ± 5.47[c]
50	37.82 ± 3.53[d]
100	18.47 ± 2.68[e]

(b)

TDB (μM)	Cell viability (%)
0	98.62 ± 1.71[a]
0.5	101.81 ± 1.77[ab]
1	98.23 ± 1.34[ab]
5	97.51 ± 8.04[ab]
10	84.14 ± 11.1[bc]
25	73.97 ± 3.55[cd]
50	61.38 ± 2.78[d]
100	38.36 ± 3.05[e]

Values are means of the independent triplicate experiments ± SD. Different small case letters in each table refer to the significant difference between means compared by one-way ANOVA with Tukey's post hoc test at α = 0.05, n = 3.

Table 4. The percent cell viability of PCS-210-010 preadipocytes (a) and adipocytes (b) after treatment with 0 - 100 μM of TDB

(a)

TDB (μM)	Cell viability (%)
0	99.33 ± 2.44[a]
0.5	100.20 ± 0.79[a]
1	100.25 ± 1.44[a]
5	99.49 ± 1.12[a]
10	90.40 ± 3.81[b]
25	56.40 ± 2.81[c]
50	39.06 ± 4.47[d]
100	19.28 ± 2.87[e]

(b)

TDB (μM)	Cell viability (%)
0	99.91 ± 3.77[a]
0.5	101.64 ± 1.66[a]
1	102.43 ± 1.41[a]
5	99.92 ± 3.74[a]
10	93.31 ± 4.89[a]
25	76.83 ± 3.67[b]
50	62.47 ± 4.18[c]
100	38.71 ± 3.34[d]

Values are means of the independent triplicate experiments ± SD. Different small case letters in each table refer to the significant difference between means compared by one-way ANOVA with Tukey's post hoc test at α = 0.05, n = 3.

Table 5. The percent Oil Red O stained after treating 3T3-L1 cells with 0 - 5 μM of TDB in the absence (-) or presence (+) of differentiation media (DIM) at early differentiation (a) and during maturation (b)

(a)

TDB (μM)	Oil Red O Staining (%)
0 (-DIM)	0 ± 0^{e}
0 (+DIM)	99.37 ± 2.27^{a}
0.5 (+DIM)	83.82 ± 1.93^{b}
1 (+DIM)	61.76 ± 5.09^{c}
5 (+DIM)	48.40 ± 2.97^{d}

(b)

TDB (μM)	Oil Red O Staining (%)
0 (-DIM)	0 ± 0^{d}
0 (+DIM)	101.82 ± 4.44^{a}
0.5 (+DIM)	95.42 ± 5.44^{a}
1 (+DIM)	77.52 ± 6.24^{b}
5 (+DIM)	63.84 ± 5.79^{c}

Values are means of the independent triplicate experiments ± SD. Different small case letters in each table refer to the significant difference between means compared by one-way ANOVA with Tukey's post hoc test at α = 0.05, n = 3.

Table 6. The percent Oil Red O stained after treating PCS-210-010 cells with 0 - 5 μM of TDB in the absence (-) or presence (+) of differentiation media (DIM) at early differentiation (a) and during maturation (b)

(a)

TDB (μM)	Oil Red O Staining (%)
0 (-DIM)	0 ± 0^{e}
0 (+DIM)	100.18 ± 1.91^{a}
0.5 (+DIM)	77.86 ± 4.78^{b}
1 (+DIM)	60.66 ± 1.40^{c}
5 (+DIM)	41.48 ± 3.81^{d}

(b)

TDB (μM)	Oil Red O Staining (%)
0 (-DIM)	0 ± 0^{d}
0 (+DIM)	98.96 ± 4.29^{a}
0.5 (+DIM)	97.17 ± 5.82^{a}
1 (+DIM)	71.87 ± 3.48^{b}
5 (+DIM)	54.93 ± 5.10^{c}

Values are means of the independent triplicate experiments ± SD. Different small case letters in each table refer to the significant difference between means compared by one-way ANOVA with Tukey's post hoc test at α = 0.05, n = 3.

Table 7. The content of cellular triglyceride after treating 3T3-L1 cells with 0 - 5 μM of TDB in the absence (-) or presence (+) of differentiation media (DIM) at early differentiation (a) and during maturation (b)

(a)

TDB (μM)	Cellular triglyceride content (mM)
0 (-DIM)	0.06 ± 0.01^{e}
0 (+DIM)	1.61 ± 0.08^{a}
0.5 (+DIM)	1.32 ± 0.06^{b}
1 (+DIM)	0.89 ± 0.09^{c}
5 (+DIM)	0.69 ± 0.07^{d}

(b)

TDB (μM)	Cellular triglyceride content (mM)
0 (-DIM)	0.05 ± 0.02^{d}
0 (+DIM)	2.25 ± 0.01^{a}
0.5 (+DIM)	2.09 ± 0.14^{ab}
1 (+DIM)	1.94 ± 0.03^{b}
5 (+DIM)	1.40 ± 0.15^{c}

Values are means of the independent triplicate experiments ± SD. Different small case letters in each table refer to the significant difference between means compared by one-way ANOVA with Tukey's post hoc test at α = 0.05, n = 3.

Table 8. The content of cellular triglyceride after treating PCS-210-010 cells with 0 - 5 μM of TDB in the absence (-) or presence (+) of differentiation media (DIM) at early differentiation (a) and during maturation (b)

(a)

TDB (μM)	Cellular triglyceride content (mM)
0 (-DIM)	0.06 ± 0.01^{e}
0 (+DIM)	1.59 ± 0.06^{a}
0.5 (+DIM)	1.35 ± 0.12^{b}
1 (+DIM)	0.98 ± 0.08^{c}
5 (+DIM)	0.60 ± 0.08^{d}

(b)

TDB (μM)	Cellular triglyceride content (mM)
0 (-DIM)	0.04 ± 0.02^{d}
0 (+DIM)	2.14 ± 0.09^{a}
0.5 (+DIM)	2.07 ± 0.07^{a}
1 (+DIM)	1.83 ± 0.06^{b}
5 (+DIM)	1.26 ± 0.07^{c}

Values are means of the independent triplicate experiments ± SD. Different small case letters in each table refer to the significant difference between means compared by one-way ANOVA with Tukey's post hoc test at α = 0.05, n = 3.

Table 9. The amount of extracellular glycerol released after treating 3T3-L1 cells with 0 - 5 μM of TDB in the absence (-) or abesence (+) of differentiation media (DIM) at early differentiation (a) and during maturation (b)

(a)

TDB (μM)	Glycerol released (mg/dL)
0 (-DIM)	0.14 ± 0.12^{e}
0 (+DIM)	1.52 ± 0.13^{d}
0.5 (+DIM)	2.33 ± 0.08^{c}
1 (+DIM)	3.18 ± 0.36^{b}
5 (+DIM)	3.96 ± 0.21^{a}

(b)

TDB (μM)	Glycerol released (mg/dL)
0 (-DIM)	0.32 ± 0.10^{d}
0 (+DIM)	2.10 ± 0.04^{c}
0.5 (+DIM)	2.18 ± 0.21^{c}
1 (+DIM)	3.39 ± 0.05^{b}
5 (+DIM)	4.74 ± 0.15^{a}

Values are means of the independent triplicate experiments ± SD. Different small case letters in each table refer to the significant difference between means compared by one-way ANOVA with Tukey's post hoc test at α = 0.05, n = 3.

Table 10. The amount of extracellular glycerol released after treating PCS-210-010 cells with 0 - 5 μM of TDB in the absence (-) or presence (+) of differentiation media (DIM) at early differentiation (a) and during maturation (b)

(a)

TDB (μM)	Glycerol released (mg/dL)
0 (-DIM)	0.14 ± 0.12^{e}
0 (+DIM)	1.38 ± 0.04^{d}
0.5 (+DIM)	2.18 ± 0.08^{c}
1 (+DIM)	3.40 ± 0.13^{b}
5 (+DIM)	4.25 ± 0.32^{a}

(b)

TDB (μM)	Glycerol released (mg/dL)
0 (-DIM)	0.29 ± 0.13^{d}
0 (+DIM)	2.08 ± 0.22^{c}
0.5 (+DIM)	2.03 ± 0.26^{c}
1 (+DIM)	3.24 ± 0.15^{b}
5 (+DIM)	4.45 ± 0.29^{a}

Values are means of the independent triplicate experiments ± SD. Different small case letters in each table refer to the significant difference between means compared by one-way ANOVA with Tukey's post hoc test at α = 0.05, n = 3.

Table 11. The percent cell proliferation and percent cell cycle progression after treating 3T3-L1 cells with 0 - 5 μM of TDB in the absence (-) or presence (+) of differentiation media (DIM) at early differentiation

(a)

TDB (μM)	Cell proliferation (%)		
	24 h	48 h	72 h
0	99.61 ± 3.38^{d}	159.54±1.42^{a}	167.99±3.48^{a}
0.5	82.91 ± 1.44^{efg}	137.89±5.72^{b}	137.84±3.91^{b}
1	79.16 ± 2.97^{fg}	125.99±6.67^{bc}	116.49±14.84^{c}
5	70.38 ± 1.56^{g}	98.69±3.66^{de}	91.03±4.28^{def}

(b)

TDB (μM)	Cell cycle progression (%)		
	G0/G1 phase	S phase	G2/M phase
0 (-DIM)	75.06±2.19^{a}	24.24±1.95	0.70±0.24$^{\gamma}$
0 (+DIM)	16.40±3.80^{d}	32.85±7.00	50.76±4.17$^{\alpha}$
0.5 (+DIM)	30.98±7.09^{cd}	39.85±2.17	29.18±7.70$^{\beta}$
1 (+DIM)	43.42±9.44^{bc}	34.59±6.08	21.99±14.71$^{\beta\gamma}$
5 (+DIM)	54.30±11.88^{ab}	36.89±6.95	8.82±4.97$^{\beta\gamma}$

Values are means of the independent triplicate experiments ± SD. Different small case letters or different Greek alphabets in each table refer to the significant difference between means compared by one-way ANOVA with Tukey's post hoc test at α = 0.05, n = 3.

Table 12. The mitotic clonal expansion (MCE)-related proteins expressions (a, b) after treating 3T3-L1 cells with 0 - 5 μM of TDB in the absence (-) or presence (+) of differentiation media (DIM) at early differentiation

(a)

TDB (μM)	Relative protein level				
	Cyclin D1	Cyclin D3	CDK2	CDK4	CDK6
0 (-DIM)	0.27±0.03^{d}	1.02±0.06^{d}	0.13±0.03^{d}	0.08±0.01^{d}	0.09±0.02^{e}
0 (+DIM)	1.15±0.10^{a}	1.79±0.09^{a}	1.13±0.07^{a}	1.18±0.03^{a}	1.19±0.06^{a}
0.5 (+DIM)	1.16±0.06^{a}	1.55±0.07^{b}	0.57±0.03^{b}	0.89±0.05^{b}	1.01±0.03^{b}
1 (+DIM)	0.94±0.03^{b}	1.34±0.04^{c}	0.48±0.03^{b}	0.78±0.07^{b}	0.84±0.07^{c}
5 (+DIM)	0.73±0.01^{c}	1.09±0.07^{d}	0.28±0.04^{c}	0.59±0.03^{c}	0.68±0.04^{d}

(b)

TDB (μM)	Relative protein level		
	p18	p21	p27
0 (-DIM)	1.27±0.07^{a}	1.21±0.03^{a}	1.27±0.07^{a}
0 (+DIM)	0.24±0.02^{e}	0.09±0.01^{e}	0.24±0.02^{e}
0.5 (+DIM)	0.50±0.02^{d}	0.22±0.01^{d}	0.50±0.02^{d}
1 (+DIM)	0.67±0.03^{c}	0.42±0.04^{c}	0.67±0.03^{c}
5 (+DIM)	0.87±0.03^{b}	1.01±0.07^{b}	0.87±0.03^{b}

Values are means of the independent triplicate experiments ± SD. Different small case letters in each table refer to the significant difference between means compared by one-way ANOVA with Tukey's post hoc test at α = 0.05, n = 3.

Table 13. The adipogenic regulators gene expressions (a) and proteins expressions (b) after treating 3T3-L1 cells with 0 - 5 μM of TDB in the absence (-) or presence (+) of differentiation media (DIM) at early differentiation

(a)

TDB (μM)	Relative mRNA level		
	Srebp1	Pparg	C/ebpa
0 (-DIM)	0.03±0.00^{e}	0.02±0.00^{d}	0.02±0.00^{e}
0 (+DIM)	1.00±0.10^{a}	1.00±0.12^{a}	1.01±0.03^{a}
0.5 (+DIM)	0.59±0.02^{b}	0.58±0.10^{b}	0.80±0.02^{b}
1 (+DIM)	0.36±0.04^{c}	0.30±0.00^{c}	0.57±0.11^{c}
5 (+DIM)	0.20±0.03^{d}	0.17±0.04^{cd}	0.22±0.03^{d}

(b)

TDB (μM)	Relative protein level		
	SREBP1	PPARγ	C/EBPα
0 (-DIM)	0.00±0.00^{e}	0.09±0.03^{d}	0.05±0.00^{e}
0 (+DIM)	0.91±0.03^{a}	1.59±0.05^{a}	1.86±0.04^{a}
0.5 (+DIM)	0.76±0.05^{b}	1.30±0.04^{b}	1.26±0.08^{b}
1 (+DIM)	0.63±0.02^{c}	0.86±0.01^{c}	0.78±0.06^{c}
5 (+DIM)	0.45±0.04^{d}	0.18±0.03^{d}	0.34±0.02^{d}

Values are means of the independent triplicate experiments ± SD. Different small case letters in each table refer to the significant difference between means compared by one-way ANOVA with Tukey's post hoc test at α = 0.05, n = 3.

Table 14. The adipogenic effectors gene expressions (a) and proteins expressions (b) after treating 3T3-L1 cells with 0 - 5 μM of TDB in the absence (-) or presence (+) of differentiation media (DIM) at early differentiation

(a)

TDB (μM)	Relative mRNA level				
	Fasn	Plin1	Lpl	Adipoq	Fabp4
0 (-DIM)	0.01 ± 0.00^{c}	0.02 ± 0.01^{c}	0.01 ± 0.00^{c}	0.02 ± 0.00^{d}	0.02 ± 0.00^{d}
0 (+DIM)	1.00 ± 0.11^{a}	1.01 ± 0.16^{a}	1.01 ± 0.16^{a}	1.00 ± 0.06^{a}	1.00 ± 0.04^{a}
0.5 (+DIM)	0.58 ± 0.12^{b}	0.48 ± 0.14^{b}	0.50 ± 0.06^{b}	0.49 ± 0.11^{b}	0.58 ± 0.09^{b}
1 (+DIM)	0.39 ± 0.07^{b}	0.35 ± 0.01^{b}	0.47 ± 0.07^{b}	0.22 ± 0.04^{c}	0.30 ± 0.10^{c}
5 (+DIM)	0.10 ± 0.02^{c}	0.25 ± 0.03^{bc}	0.12 ± 0.04^{c}	0.10 ± 0.01^{cd}	0.16 ± 0.05^{cd}

(b)

TDB (μM)	Relative protein level				
	FAS	PLIN1	LPL	Adiponectin	FABP4
0 (-DIM)	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	0.01 ± 0.00^{e}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}
0 (+DIM)	0.77 ± 0.04^{a}	0.42 ± 0.02^{a}	0.68 ± 0.01^{a}	0.43 ± 0.02^{a}	0.38 ± 0.02^{a}
0.5 (+DIM)	0.62 ± 0.03^{b}	0.36 ± 0.01^{b}	0.60 ± 0.04^{b}	0.24 ± 0.02^{b}	0.19 ± 0.03^{b}
1 (+DIM)	0.30 ± 0.02^{c}	0.17 ± 0.02^{c}	0.40 ± 0.03^{c}	0.06 ± 0.00^{c}	0.05 ± 0.01^{c}
5 (+DIM)	0.06 ± 0.01^{d}	0.10 ± 0.00^{d}	0.29 ± 0.02^{d}	0.01 ± 0.00^{d}	0.00 ± 0.00^{d}

Values are means of the independent triplicate experiments ± SD. Different small case letters in each table refer to the significant difference between means compared by one-way ANOVA with Tukey's post hoc test at α = 0.05, n = 3.

Table 15. The adipogenic effectors gene expressions (a) and proteins expressions (b) after treating 3T3-L1 cells with 0 - 5 μM of TDB in the absence (-) or presence (+) of differentiation media (DIM) during maturation

(a)

TDB (μM)	Relative mRNA level				
	Fasn	Plin1	Lpl	Adipoq	Fabp4
0 (-DIM)	0.01 ± 0.00^{e}	0.01 ± 0.01^{c}	0.01 ± 0.00^{d}	0.05 ± 0.04^{c}	0.02 ± 0.01^{d}
0 (+DIM)	1.01 ± 0.07^{a}	1.00 ± 0.11^{a}	1.01 ± 0.16^{a}	1.01 ± 0.21^{a}	1.00 ± 0.06^{a}
0.5 (+DIM)	0.82 ± 0.06^{b}	0.48 ± 0.06^{b}	0.69 ± 0.09^{b}	0.58 ± 0.08^{b}	0.65 ± 0.14^{b}
1 (+DIM)	0.58 ± 0.11^{c}	0.17 ± 0.05^{c}	0.36 ± 0.02^{c}	0.34 ± 0.12^{bc}	0.47 ± 0.05^{b}
5 (+DIM)	0.22 ± 0.03^{d}	0.09 ± 0.04^{c}	0.18 ± 0.03^{cd}	0.19 ± 0.08^{c}	0.24 ± 0.05^{c}

(b)

TDB (μM)	Relative protein level				
	FAS	PLIN1	LPL	Adiponectin	FABP4
0 (-DIM)	0.01 ± 0.00^{d}	0.01 ± 0.00^{e}	0.00 ± 0.00^{e}	0.11 ± 0.00^{d}	0.03 ± 0.00^{d}
0 (+DIM)	0.86 ± 0.04^{a}	0.92 ± 0.06^{a}	0.93 ± 0.07^{a}	1.08 ± 0.04^{a}	0.95 ± 0.05^{a}
0.5 (+DIM)	0.46 ± 0.08^{b}	0.71 ± 0.05^{b}	0.75 ± 0.04^{b}	0.84 ± 0.04^{b}	0.80 ± 0.06^{b}
1 (+DIM)	0.19 ± 0.04^{c}	0.44 ± 0.04^{c}	0.45 ± 0.03^{c}	0.49 ± 0.04^{c}	0.44 ± 0.03^{c}
5 (+DIM)	0.02 ± 0.00^{d}	0.29 ± 0.03^{d}	0.24 ± 0.01^{d}	0.41 ± 0.00^{c}	0.09 ± 0.01^{d}

Values are means of the independent triplicate experiments ± SD. Different small case letters in each table refer to the significant difference between means compared by one-way ANOVA with Tukey's post hoc test at α = 0.05, n = 3.

Table 16. Protein expressions of phosphorylated proportions involved in some adipocyte differentiation-linked biological pathways after treating 3T3-L1 cells with 0 - 5 μM of TDB in the absence (-) or presence (+) of differentiation media (DIM) at early differentiation (a,b)

(a)

TDB (μM)	Relative protein level	
	p-AKT/AKT	p-GSK-3β/GSK-3β
0 (-DIM)	0.44±0.02^{d}	0.31±0.02^{d}
0 (+DIM)	1.15±0.05^{a}	0.80±0.03^{a}
0.5 (+DIM)	0.92±0.02^{b}	0.48±0.03^{b}
1 (+DIM)	0.86±0.06^{bc}	0.45±0.02^{bc}
5 (+DIM)	0.78±0.03^{c}	0.40±0.04^{c}

(b)

TDB (μM)	Relative protein level		
	p-AMPKα/AMPKα	p-AMPKβ1/AMPKβ1/2	p-ACC/ACC
0 (-DIM)	1.03±0.01^{a}	1.40±0.07^{a}	1.17±0.08^{a}
0 (+DIM)	0.57±0.01^{d}	0.33±0.03^{d}	0.31±0.03^{e}
0.5 (+DIM)	0.61±0.02^{d}	0.64±0.03^{c}	0.48±0.04^{d}
1 (+DIM)	0.81±0.03^{c}	0.75±0.04^{c}	0.67±0.02^{c}
5 (+DIM)	0.93±0.01^{b}	1.05±0.05^{b}	0.88±0.02^{b}

Values are means of the independent triplicate experiments ± SD. Different small case letters in each table refer to the significant difference between means compared by one-way ANOVA with Tukey's post hoc test at α = 0.05, n = 3.

Table 17. Protein expressions of phosphorylated proportions involved in some adipocyte differentiation-linked biological pathways after treating 3T3-L1 cells with 0 - 5 μM of TDB in the absence (-) or presence (+) of differentiation media (DIM) during maturation (a,b)

(a)

TDB (μM)	Relative protein level	
	p-AKT/AKT	p-GSK-3β/GSK-3β
0 (-DIM)	0.44±0.09^{d}	0.34±0.03^{e}
0 (+DIM)	1.08±0.05^{a}	0.87±0.02^{a}
0.5 (+DIM)	0.85±0.02^{b}	0.73±0.01^{b}
1 (+DIM)	0.80±0.01^{bc}	0.63±0.03^{c}
5 (+DIM)	0.66±0.04^{c}	0.50±0.05^{d}

(b)

TDB (μM)	Relative protein level		
	p-AMPKα/AMPKα	p-AMPKβ1/AMPKβ1/2	p-ACC/ACC
0 (-DIM)	1.52±0.15^{a}	1.29±0.09^{a}	1.45±0.05^{a}
0 (+DIM)	0.49±0.04^{d}	0.66±0.03^{d}	0.33±0.06^{d}
0.5 (+DIM)	0.51±0.06^{d}	0.87±0.07^{c}	0.52±0.02^{c}
1 (+DIM)	0.85±0.04^{c}	1.05±0.04^{b}	1.26±0.04^{b}
5 (+DIM)	1.21±0.01^{b}	1.09±0.07^{b}	1.30±0.06^{b}

Values are means of the independent triplicate experiments ± SD. Different small case letters in each table refer to the significant difference between means compared by one-way ANOVA with Tukey's post hoc test at α = 0.05, n = 3.

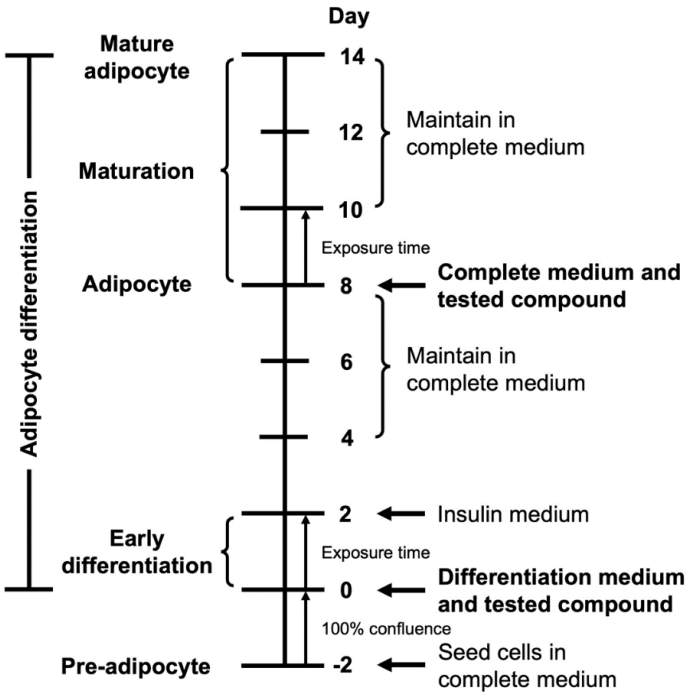


Figure 23. Schematic diagram demonstrating different phases in the adipocyte differentiation course and the experimental design conducted in this study. The bioactivities of the tested compound, 4,5,4′-trihydroxy-3,3′-dimethoxybibenzyl, were assessed in early differentiation and maturation. The compositions of media and detailed experimental procedures are addressed in experimental section.

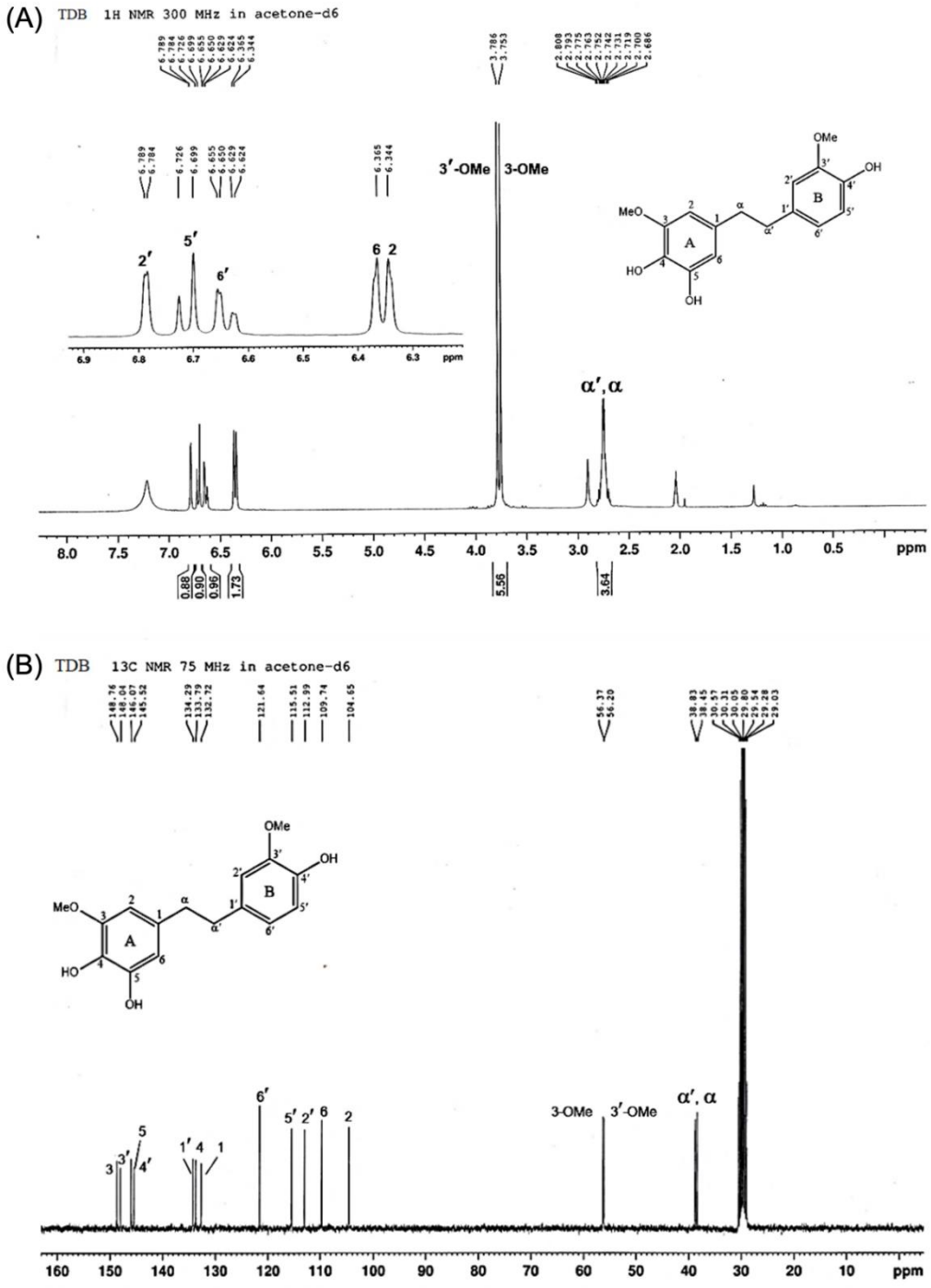


Figure 24. Structural elucidation with nuclear magnetic resonance (NMR) spectra of 4,5,4′-trihydroxy-3,3′-dimethoxybibenzyl (TDB). ^{1}H-NMR (300 MHz) (A) and ^{13}C-NMR (75 MHz) (B) spectra of TDB (in acetone-d_6).

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AWARD RECEIVED	(1) VICHIARA JIRAWONGSE AWARD for the Graduate Research Excellence in Herbal Medicine 2022 (2) Best poster (First) 2020, Pharmaceutical Sciences and Technology program, Faculty of Pharmaceutical Sciences, Chulalongkorn University