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EFFECT OF COMBINATION OF SAHA AND THA ON KEY METABOLIC REGULATORY PROTEINS AND ADIPOCYTE APOPTOSIS IN 3T3L1 PRE-ADIPOCYTIC CELL LINE

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ABSTRACT: Obesity is a state characterized by excessive storage of triglycerides in the adipose tissue that leads to the adverse medical consequence. The increasing prevalence and morbidity associated with the disease makes it a serious issue of concern. The treatment regimens for the obesity include diet plans, increase in physical activity, anti-obesity medications and surgery. However, the treatment of obesity by lifestyle modifications alone is complicated, requiring therapy with drugs, but the side effects associated with available drugs (Orlistat & Sibutramine) have become a crucial drawback, demanding the discovery of new molecules targeting multiple pathways involved in the pathogenesis of obesity. Combinational therapies are well suited for the pathological conditions where many regulatory mechanisms lead to the development and the progression of the disease. In our present study, we investigated the effects of combination of Suberoylanilide hydroxamic acid (SAHA) and O2′, O3′, O5′-tri-acetyl-N6-(3-hydroxylaniline) adenosine (THA) in *in vitro* model of Obesity. The results showed that combination of test compounds synergistically enhanced the expression levels of phosphorylated AMPK (AMP-activated protein kinase). The expression levels of key proteins regulating fatty acid synthesis and oxidation were also modulated. The Western blot analysis revealed a significant reduction in the protein expression levels of fatty acyl synthase (FAS) and increased expression levels of Carnitine Palmitoyl Transferase (CPT-1) protein. Furthermore, the co-treatment of drugs induces the adipocyte apoptosis as depicted from Propidium Iodide staining. The results in totality suggest that the combinational therapy targets key metabolic regulatory pathways like fatty acid synthesis and oxidation, energy homeostasis and adipocyte apoptosis implicating the relevance of therapeutically exploiting the combination treatment for the management of obesity. The combination of SAHA and THA seem to have promising benefits for the treatment and prevention of obesity.

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KEYWORDS: Obesity, Combitorial therapy, AMP-activated Protein Kinase, Propidium Iodide, pre-adipocytic cell line.

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1. INTRODUCTION

Obesity is a chronic condition which arises due to energy imbalance in the body [1]. The imbalance occurs when the energy consumption exceeds the energy utilisation by the body leading to increase in fat deposits in the adipose tissue and liver [2]. The excess energy stored leads to either increase in number of fat cells (hyperplasia) or increase in the size of fat cells (hypertrophy). With time the increase in energy and fat reserves leads to Obesity [3]. It is not only a single disorder but associated with serious metabolic consequences like Type 2 Diabetes, hypertension, atherosclerosis, arthritis, sleep apnoea, cardiovascular diseases, liver abnormalities, psychosocial dysfunction and even cancers. The clinical manifestations include increase in the levels of triglycerides, cholesterol, free fatty acids, blood glucose and insulin resistance whereas decrease in the levels of adiponectin and high density lipoproteins and endocrine changes [4].The Body Mass Index, skin fold thickness and waist circumference are different parameters taken into consideration while classifying individuals as normal, overweight and obese. The individuals who have BMI greater than 25 kg/m^2 are considered as overweight and are at risk of developing the serious metabolic abnormalities associated with it. The individuals with BMI greater than $30kg/m^2$ are considered as obese and are at high risk of obesity linked consequences [5-7]. Waist circumference is used to assess the overweight and obesity and measured at the midpoint between the upper border of the pelvis and lower border of the ribs. For men, the waist circumference $\geq$94 cm and $\geq$102 cm represent the increased risk and substantially increased risk of metabolic complications respectively. However, for women, the waist circumference $\geq$88cm represents a higher risk of metabolic complications [8].The measurement of skin fold thickness at multiple sites with the help of callipers provides a more precise assessment [9].The physical activity programs, diet plans and lifestyle modifications are the prime component of treatment regimens whereas drugs and surgery are the other options for combating the disease [10]. Achievement of tight serum lipid control by diet alone is difficult, requiring therapy with drugs. The side effects associated with available drugs have become a limiting factor, demanding the discovery of new molecules targeting various pathways involved in the pathogenesis of obesity [11]. Several strategies have been used to design anti-obesity medications. The strategies were aimed to increase energy expenditure, appetite suppression, targeting fat metabolism, inhibiting adipocyte

differentiation etc [12]. Based on these strategies, several drugs were developed but only Orlistat and Sibutramine were recommended for treatment purposes. Orlistat is a lipase inhibitor and targets fat metabolism whereas Sibutramine is a β-phenethylamine that selectively inhibits the reuptake of noradrenaline, serotonin and acts by appetite suppression mechanism. Both of these drugs have shown severe side effects like gastrointestinal disorders, cardiovascular disorders etc eliciting an immediate need to design novel strategies for combating the problem of Obesity [13-15]. Since obesity like hypertension is a complex diseases which arises due to multifactorial reasons, so using combination of drugs might prove effective in its treatment [16, 17]. So we used two test compounds SAHA and THA in our study. THA is a synthetic AMPK activator whereas SAHA is an HDAC inhibitor. AMPK is a heterotrimeric protein involved in regulating energy homeostasis by having a direct role in carbohydrate, protein and lipid metabolism and even adipocyte differentiation. It is the prime target and potential candidate for Obesity treatment [18, 19]. HDAC inhibitors are promising class of therapeutic agents against inflammation, neurodegenerative and metabolic disorders. They regulate gene expression and thereby control cell proliferation, migration and death. SAHA has shown inhibitory effect on adipogenesis in pre-adipocytic cell lines and anti-inflammatory effect [20, 21]. So, by selecting the combination of SAHA and THA, we aimed to target fat metabolism, adipocyte differentiation and obesity-associated inflammation all at once. In our previous study, these two test compounds showed synergistic effect in reduction in the viability of mature adipocytes, enhanced inhibition of adipogenesis and reduction in lipid content in mature adipocytes. There was significant reduction in the expression levels of transcriptional factors (Peroxisome Proliferator Activated Receptor-γ and CCAAT/Enhancer Binding Protein-α), involved in regulating differentiation of pre-adipocytes into mature adipocytes. The combination also induced apoptosis in mature adipocytes possibly by increasing expression levels of Bcl-2 Associated X-protein [22]. Thus the present study was aimed to study effect of combination of test compounds on the key metabolic regulatory proteins and on adipocyte apoptosis.

2. MATERIALSAND METHODS

2.1. Reagents:

Dulbecco's modified Eagles medium (DMEM) and Fetal Calf Serum (FCS) were purchased from GIBCO (St. Louis, MO, USA).The SAHA drug, Isobutylmethylxanthine (IBMX), were purchased from Sigma Aldrich (St. Louis, MO, USA). The antibodies for AMP-activated protein Kinase (AMPK), Fatty Acyl Synthase (FAS) and Carntine Palmitoyl Transferase 1 (CPT-1) and Tubulin were obtained from Santa Cruz Biotechnologies, (CA, USA) whereas secondary anti-rabbit and anti-mouse antibodies were purchased from Cell Signalling Technologies (Beverly, MA).The O2′, O3′, O5′-tri-acetyl-N6-(3-hydroxylaniline) adenosine (THA) drug was synthesized by our collaborators from CSIR-Indian Institute of Integrative Medicine (IIIM) Jammu.

2.2. Cell culture:

3T3-L1 mouse embryonic fibroblasts were obtained from National Centre For Cell Science (Pune, India), maintained at 37°C in a humidified 5% CO_2 atmosphere and cultured as per standard protocol [23]. Briefly, cells were cultured in Dulbecco's modified Eagles medium (DMEM) containing 10% (v/v) Fetal Calf Serum (FCS), 100 U/ml of penicillin and 100 µg/ml of streptomycin, until confluent. Two days after confluence (Day 0), the cells were stimulated to differentiate with differentiation media (DI media) consisting of DMEM, 10% FCS, 167 nM insulin, and 0.5 µM Isobutylmethylxanthine and 1 µM dexamethasone for two days (Day 2). The differentiation media was replaced by DMEM+10% FCS+ 167 nM insulin for next two days (Day 4), followed by culturing with DMEM+10% FCS for additional 4 days (Day 8), at which about 90% of cells were found to be mature adipocytes with fat droplets.

2.3. Western blot Analysis:

For immunoblotting, the test compounds were added along with the differentiation media and the differentiation of 2-day post confluent 3T3-L1 cells was carried out as described under "cell culture". On Day 6, cells were harvested, washed with phosphate-buffered saline (PBS) and suspended in NP-40 lysis buffer [20 mM Tris Cl (pH 8), 137 mM NaCl, 10% Glycerol, 1% Nonidet P-40, and 2mM EDTA] for one hour. The supernatant was separated by centrifugation (12,000 g for 10 min) and the protein concentration was estimated using Bradford's assay [24]. Protein extract, preheated at 100°C for 5 min in reducing SDS sample buffer containing 50 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 0.1% bromophenol blue, 100 mM β-mercaptoethanol, was run on 10% SDS-polyacrylamide gel. After gel electrophoresis, separated proteins were transferred to PVDF membrane by semi-dry transfer method in accordance with the manufacturer's instructions (Hoefer-USA). The membranes were blocked with 5% bovine serum albumin (BSA) for 3 h, washed twice with PBS containing 0.2% Tween-20, and incubated with the respective primary antibodies [AMPK (1: 1000 dilution), FAS (1:1000 dilution), CPT-1 (1:2000 dilution) and Tubulin (1:1000 dilution)] overnight at 4°C. The secondary detection was performed using anti-mouse IgG DyLight® 680 conjugate (1:10,000) or anti-rabbit IgG DyLight® 800 conjugate (1:10,000) secondary antibodies. The fluorescence was detected using Odyssey infrared detection system. Quantitative analysis of immunoblots was performed by densitometry (normalised with Tubulin).

2.4. Propidium iodide staining

Propidium iodide [PI] is an indicator of apoptosis. It penetrates only damaged cells and intercalates into double-stranded DNA in a stoichiometric manner. The permeability of cell membrane is increased when the cell suffers damage and loses its membrane integrity. PI as a result is incorporated into cell and binds to DNA. Positive staining of the nuclei thus indicates loss of membrane integrity and thus is an indication of apoptosis. For PI staining, the mature 3T3-L1 cells were treated with SAHA and THA alone and in combination for 24 hours. After aspirating the media, the cells were

washed gently twice with pre-chilled PBS for 5 min. Cells were then incubated with 5 µg/ml PI solution for 30 min in the dark. This was followed by washing with Tris buffer (50 mM Tris-HCl, pH7.3). Stained cells were monitored using the Floid Cell Imaging Station (Life Technologies).

2.5. Statistical analysis

Results represent means ± SD of three independent experiments. Statistical analysis was performed using One Way ANNOVA by Dunnett's multiple comparison tests utilizing Graphpad prism 5 software. Statistically significant differences are defined at the 95% confidence interval (***P<.001, **P<.01).

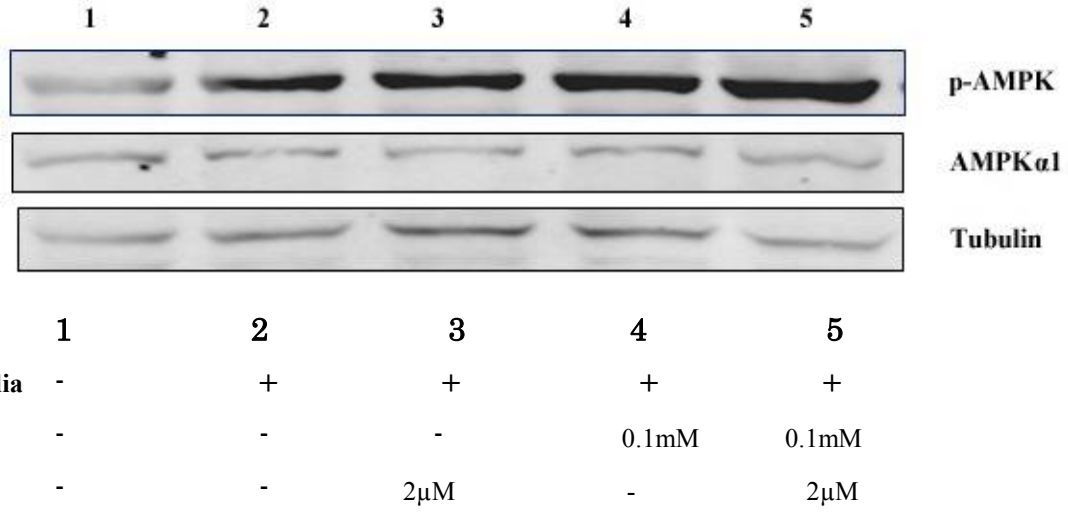
3. RESULT AND DISCUSSION

3.1. Combination of SAHA and THA promotes fatty acid oxidation by activating AMP-activated protein kinase (AMPK).

AMPK has been proposed to act as a "metabolic master switch" mediating the cellular adaptation to environmental or nutritional stress factors. The activation of AMPK by phosphorylation is known to play crucial role in fatty acid oxidation by regulating mitochondrial availability of fatty acids. Once activated, AMPK leads to a concomitant inhibition of energy-consuming biosynthetic pathways, such as fatty acid and sterol synthesis, and activation of ATP-producing catabolic pathways, such as fatty acid oxidation. Phosphorylated AMPK phosphorylates (inactivates) ACC (Acetyl Co A Carboxylase) and lowers the intracellular malonyl-CoA level, which is the substrate for fatty acid synthesis and, at the same time, the inhibitor of Carnitine palmitoyl transferase-1 (CPT-1), the rate-limiting enzyme of mitochondrial fatty acid oxidation

To know effect of combination of SAHA and THA on the expression levels of phosho-AMPK and AMPK α1, western blot analysis for these two proteins was done. 3T3-L1 pre-adipocytes were treated with test compounds alone and in combination from Day 0 to Day6 during the differentiation process. Total cell lysates were subjected to immunoblotting using antibodies specific for phospho-AMPK (Thr172), AMPKα1 and Tubulin. The immunoblotting results indicated a significant increase in the expression levels of AMPK, however no detectable change in the expression of AMPK α1 was observed [Fig 3.1(A)] when the test compounds were used in combination. Tubulin was used as an internal control reference of sample loading. Quantitative analysis of immunoblots performed by densitometry showed significant differences when combination of SAHA and THA was used as represented in Bar 3.1(B).

(A)



	1	2	3	4	5
DI Media	-	+	+	+	+
THA	-	-	-	0.1mM	0.1mM
SAHA	-	-	2µM	-	2µM

(B)

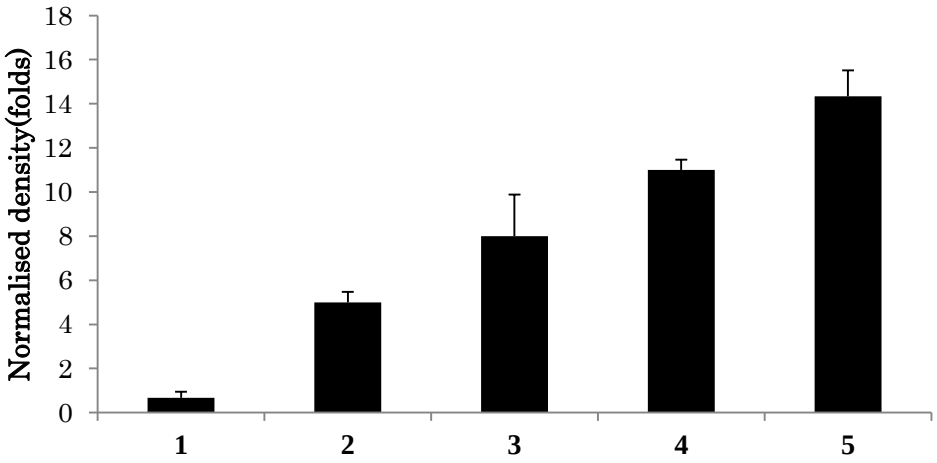


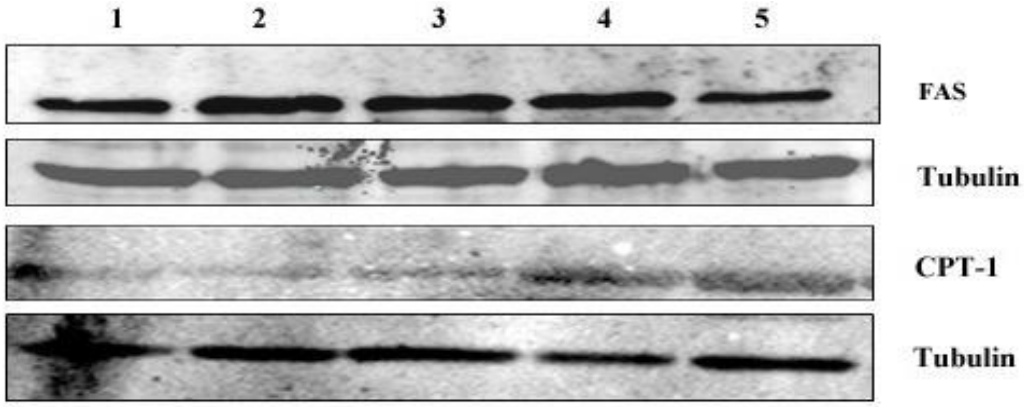
Fig 3.1: Effect of combination of SAHA and THA on the phospho-status of AMPK. Differentiated 3T3-L1 adipocytes were treated with DMSO and the test compounds alone and in combination. The immunoblots for p-AMPK, AMPKα1 in response to control versus treated cells reveals synergistic effect of combination of test compounds in activating AMPK as shown in Fig (A). The quantitative analysis of immunoblots relative to loading control is shown in (B).

3.2. Combination of SAHA and THA modulate the expression levels of key metabolic enzymes: Since, the adipogenic transcription factors were down-regulated by the combination of test compounds; we further studied the expression patterns of their downstream protein targets such as Fatty acyl synthase (FAS) and Carnitine palmitoyltransferase I (CPT1). FAS is a multienzyme protein that catalyzes fatty acid synthesis from acetyl-CoA and malonyl-CoA into long-chain fatty acids, and also is increased during adipogenesis process. FAS catalyzes the synthesis of saturated fatty acids,

predominately palmitate, from acetyl-CoA and malonyl-CoA precursors. This enzyme is highly expressed in the liver, adipose tissue, and lactating mammary glands. Malonyl- CoA generated by ACC1 is utilized by FAS for the synthesis of fatty acids in the cytosol and it plays important roles in the development of hepatic steatosis and insulin resistance. CPT-1 is a rate-limiting enzyme in beta-oxidation of fatty acids and is used as a marker of lipid degradation. CPT-1 catalyses one of the essential steps in the beta oxidation of long chain fatty acids and determines not only the absolute rate of fatty acid oxidation, but also the cytosolic concentration of long-chain acyl-Co A esters. Its importance in fatty acid metabolism makes CPT1 a potentially useful enzyme to focus on in the development of treatments of many other metabolic disorders as well.

To know the effect of combinational drug therapy on the protein expression levels of key metabolic enzymes like Fatty acyl Synthase (FAS) and CPT-1, the whole cell lysates were prepared and after protein quantification, western blotting was done. Treatment of 3T3-L1 cells with combination of SAHA and THA showed downregulation of FAS and up regulation of CPT-1 protein compared to individual drug concentrations [Fig 3.2(A) & (B) respectively]. Tubulin was used as loading control. Quantitative analysis performed by densitometry of immunoblots showed significant differences when combination of drugs was used (Bar graph 3.2C and 3.2.D.

(A)



(B)

	1	2	3	4	5
DI Media	-	+	+	+	+
THA	-	-	0.1mM	-	0.1mM
SAHA	-	-	-	2µM	2µM

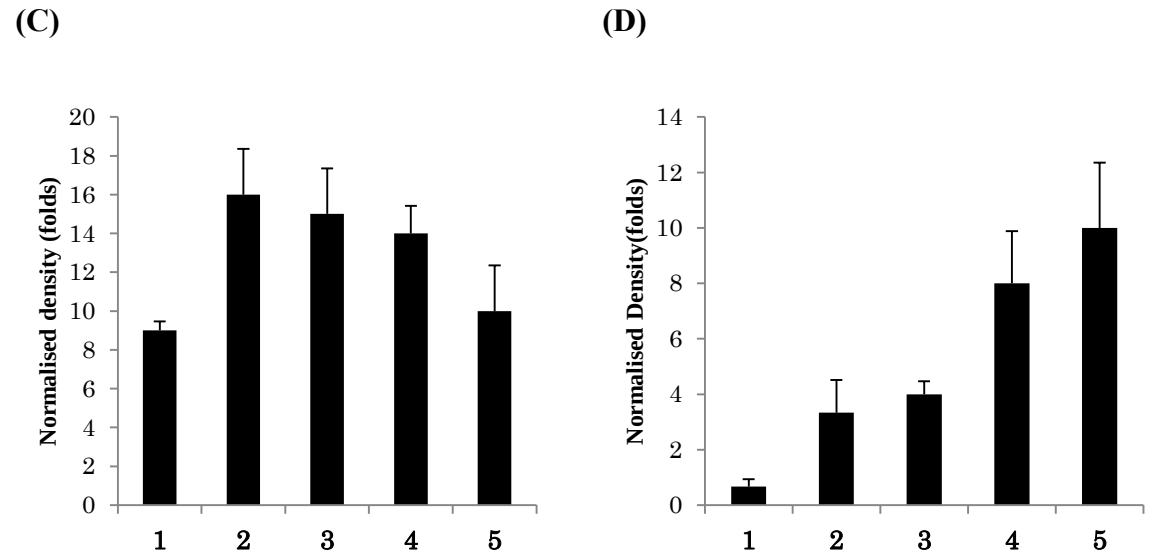


Fig 3.2: Effect of combination of SAHA and THA the expression levels of key metabolic enzymes: The immunoblots for FAS and CPT1 in response to control vs treated cells are shown in Fig (A) and (B) whereas quantitative analysis of immunoblots is shown in (C) and (D) respectively.

3.3. Combination of SAHA and THA induces adipocyte apoptosis

The process of adipogenesis is a critical phenomenon regulating the development of a chronic disease, i.e. obesity. The adipose tissue mass can be reduced either by inhibiting adipogenesis or by inducing apoptosis of adipocytes. So, in order to evaluate the effect of drug treatment on the process of adipocyte apoptosis, we carried out PI staining. PI is an index of cell damage and apoptosis under non fixed cell staining conditions thus delineating morphological changes induced by stress from changes in morphology due to apoptosis. Apoptotic changes were analysed via observation of cellular and nuclear morphological changes.

The results presented in Fig 3.1.5 showed that treatment of 3T3-L1 adipocytes with SAHA and THA in combination significantly induce cell apoptosis in comparison to control group. 3T3-L1 cells treated with combination displayed morphological changes typical of cells undergoing apoptosis. In the control group, nuclei stained with PI were fewer in comparison to drug treated group, but the combination treated cells showed marked granular apoptotic bodies Fig 3.3(E). Higher percentages of cell damage were induced by combination treatment in comparison to other groups. In order to know the effect on normal fibroblasts, NIH-3T3-L1 cells were used and it was seen that the combination of drugs showed no significant effect on normal cells implying that the test compounds induce apoptosis of adipocytes selectively Fig 3.3(F). Thus from PI staining, we concluded that combination drug treatment induces apoptosis of 3T3-L1 adipocytes. The lower doses of test

compound in combination also showed higher apoptotic bodies (D) compared to control group, implying synergistic effect of both the compounds.

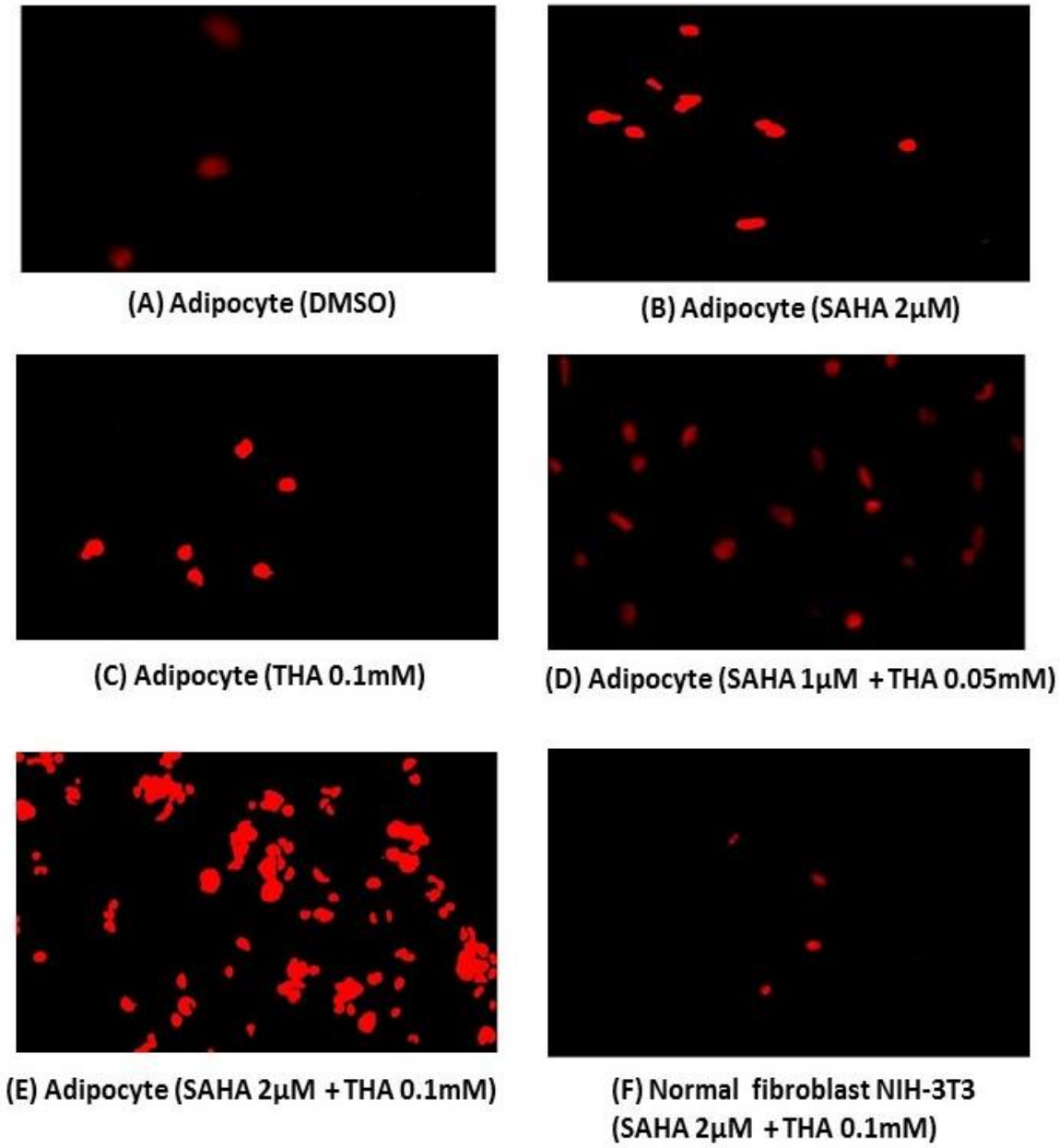


Fig 3.3: Effect of combination of SAHA and THA on the induction of apoptosis in mature adipocytes. Representative photomicrographs showing fluorescent images stained with Propidium Iodide as an index of apoptosis. The combination treated cells showed maximum marked granular apoptotic bodies (E) in comparison to the control DMSO treated adipocytes (A). The NIH-3T3 cells were used to check the effect of combination of drugs on normal fibroblasts (F).

4. CONCLUSION

Obesity is a serious health and economic burden and has reached pandemic proportions [25]. The increasing prevalence and multiple health risks associated with obesity demand an urgent need to combat the disease. Currently, only a few approved obesity pharmacological treatments are available due to their modest efficacy and safety concerns [26]. For a multifactorial disease like obesity,

combinatorial therapies are thought to provide more beneficial outcome compared to monotherapies. They may synergistically increase or decrease the therapeutic potential or toxicity of drugs. Because combination therapies are often used to treat chronic diseases, we investigated the effect of the combination of test compounds Suberoylanilide hydroxamic acid (SAHA) and O2', O3', O5'-tri-acetyl-N6-(3-hydroxylaniline) adenosine (THA) in *in vitro* conditions. So, by choosing the combination of SAHA and THA, we intended to target fat metabolism, adipocyte differentiation and obesity-associated inflammation all at once. The increase in adipose tissue mass is determined by the number and size of adipocytes which depends upon the proliferation and differentiation of preadipocytes into adipocytes .Therefore, the growth and proliferation of preadipocytes have a profound implication in the development of obesity therapeutics. To know the effect of test compounds on adipocyte differentiation, 3T3-L1 pre-adipocytic cell line was used as an *in vitro* model for the study. The pre-adipocytes were stimulated to differentiate into mature adipocytes by using a standard adipogenic medium that contains insulin, isobutylmethylxanthine and dexamethasone. We investigated the effects of combination treatment on the phosphorylation status of AMPK, which acts as a master regulator of energy metabolism. The activation of AMPK by phosphorylation directly modulates fatty acid synthesis and oxidation by altering the expression and activation of enzymes and proteins involved in fat metabolism. AMPK also regulates preadipocyte differentiation and adipogenesis. With the increase in the evidence regarding vital role that AMPK plays as an intracellular energy sensor, it has become of great interest in recent years as molecular targets in obesity research. In this study, SAHA and THA when used in combination at the concentration of SAHA 2µM and THA 0.1mM significantly increased the phosphorylation status and thereby activation of AMPK as compared to individual treatments. Since phosphorylated form of AMPK is the activated form, this points to the fact that combination treatment activates it.While dealing with the problem of obesity we had to look for the different enzymes which became the reason for recruitment of primary fodder for triacylglycerols i.e. the fatty acids. For this, the protein expression patterns of Fatty acyl synthase (FAS) and Carnitine palmitoyl transferase 1(CPT1) were studied by immunoblotting. FAS is the key enzyme controlling fatty acids synthesis whereas CPT1 transports long-chain fatty acids into mitochondria for beta-oxidation and thus acts as a vital regulatory enzyme in modulating fatty acid oxidation. The results showed significant downregulation of FAS and upregulation of CPT1 protein by using a combination of SAHA and THA rather than when these compounds were used individually. The results clearly depicted the treatment potential of test compounds by affecting fat metabolism via modulating the expression patterns of regulatory proteins. Thus, the data obtained from the *in vitro* studies showed that SAHA and THA act synergistically to inhibit adipogenesis, induce adipocyte apoptosis, activate the fatty acid oxidation and inhibit the fatty acid synthesis. The results from in vitro model were promising and lead us to evaluate the treatment therapy in in vivo model.

Propidium iodide staining was carried out to investigate the effect of test compound on the process of apoptosis. The cells were first differentiated, treated with the test compound alone and in combination and stained with propidium iodide. The images captured were analysed, and it was inferred that the test compounds induce the apoptosis of adipocytes. The combination treated cells showed maximum marked granular apoptotic bodies in comparison to single test compound treated cells. Moreover, an interesting observation was that the normal fibroblasts (NIH-3T3 cells) on treatment with a combination of test compounds showed no significant granular apoptotic bodies compared to adipocytes. Thus, the results obtained from both the previous and present studies in in vitro models of obesity show that the combination of SAHA and THA act may prove effective strategy to combat obesity. There was synergistic inhibition of adipogenesis, induction of apoptosis in adipocytes, inhibition of fatty acid synthesis, promotion of fatty acid oxidation, implicating the relevance of considering these compounds for the treatment of obesity. However, further in vivo studies need to be carried out to validate the therapeutic potential of the combination of SAHA and THA against obesity. Consequently, we speculate that the treatment with combination of SAHA and THA be an active medication to treat obesity and obesity-related metabolic diseases in the future.

CONFLICT OF INTEREST: None

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