

A 1,2,3-Triazole Derivative Mitigates Palmitic Acid-Induced Lipotoxicity Through Coordinated Modulation of Lipogenic, Oxidative, and Inflammatory Pathways in HepG2 Cells

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Abstract

Metabolic dysfunction-associated steatotic liver disease (MASLD) is characterised by excessive lipid accumulation in hepatocytes, oxidative stress, mitochondrial dysfunction and inflammation, yet effective therapeutic options remain limited. Here, we investigated the protective effects of a novel 1,2,3-triazole derivative (molecule **3**) in a palmitic acid (PA)-induced lipotoxicity model using HepG2 cells. Exposure to PA induced intracellular lipid accumulation (steatosis), oxidative stress, mitochondrial dysfunction, apoptosis and inflammatory responses, recapitulating key features of hepatocellular lipotoxicity. Treatment with molecule **3** markedly reduced lipid accumulation and oxidative stress, preserved mitochondrial integrity and membrane potential, and attenuated apoptotic cell death. In this study, molecule **3** also modulated lipid-metabolism gene expression, enhancing genes associated with metabolic homeostasis while suppressing lipogenic signalling. In parallel, it reduced the expression of pro-inflammatory mediators linked to lipotoxic injury. These findings demonstrate that molecule **3** protects hepatocytes against palmitic acid-induced lipotoxicity by coordinating the modulation of metabolic, oxidative, and inflammatory pathways. The results identify molecule **3** as a promising hepatoprotective agent that warrants further evaluation in advanced preclinical models of MASLD.

1. Introduction

Metabolic-dysfunction-associated steatotic liver disease (MASLD) has emerged as a pressing global epidemic, largely fuelled by the rising prevalence of metabolic syndrome [1, 2]. This condition is not only responsible for increasing rates of liver-related morbidity and mortality but is also a leading contributor to hepatocellular carcinoma (HCC), with an alarming 38% of cases attributed to it. MASLD accounts for over 90% of primary liver tumours and stands as the fifth most common cancer, as well as the third leading cause of cancer-related deaths worldwide [3, 4]. The progression of MASLD is concerning, starting from the accumulation of triglycerides ($\geq 5\%$) in liver cells, known as simple steatosis, and advancing to more severe stages such as metabolic dysfunction-associated steatohepatitis (MASH), fibrosis, cirrhosis, and ultimately HCC [5]. The prevalence of MASLD is striking, affecting 25–38% of the global population [6]. Its close association with insulin resistance, obesity, type 2 diabetes mellitus, and dyslipidaemia underscore the urgent need for effective interventions, as it reflects a continuum of escalating clinical severity that demands immediate attention and action [7, 8]. At its core, MASLD is driven by a complex interplay of factors, including hepatic lipid overaccumulation, mitochondrial dysfunction, oxidative stress, endoplasmic reticulum stress, and hepatic inflammation [4, 9]. Historically, this intricate pathophysiological framework has been encapsulated in the "multiple-hit" hypothesis [10]. The pivotal factor in the onset of MASLD is an imbalance between lipid accumulation and lipid utilisation, culminating in excessive lipid accumulation, characterised by elevated circulating free fatty acids, especially saturated fatty acids such as palmitic acid (PA) [11, 12]. PA serves as a primary catalyst of hepatocyte injury, triggering lipotoxicity through several mechanisms: activation of de novo lipogenesis, disruption of mitochondrial oxidative phosphorylation, generation of reactive oxygen species (ROS), and induction of pro-apoptotic and pro-inflammatory signalling pathways [13, 14]. This molecular landscape is further complicated by the dysregulation of key metabolic sensors, including sterol

regulatory element-binding protein-1c (SREBP-1c), carbohydrate-responsive element-binding protein (ChREBP), acetyl-CoA carboxylase (ACC), stearyl-CoA desaturase 1 (SCD1), cluster of differentiation 36 (CD36), peroxisome proliferator-activated receptor- α (PPAR α), small heterodimer partner (SHP), and cytochrome P450 7A1 (CYP7A1) [15–23]. Despite extensive research over the decades, the pharmacological mechanisms driving MASLD remain elusive, particularly as its global prevalence continues to rise. Presently, only two pharmacological treatments have received approval: resmetirom, a thyroid hormone receptor- β agonist, and semaglutide [24, 25]. Both have specific indications for MASH, yet they come with significant limitations, including partial efficacy, adverse side effects, and high costs [26, 27]. This glaring unmet medical need highlights the urgent necessity to discover novel chemical entities with unique mechanisms of action that can effectively target the multifaceted pathogenesis of MASLD [28]. A critical driver of the progression from simple steatosis to advanced stages of MASLD is hepatic inflammation [29]. Pathways involving TNF- α , IL-6, IL-12, and IFN- γ have emerged as key orchestrators of hepatic immunological dysregulation and disease progression. These inflammatory mediators are crucial in driving the transition from early steatosis to advanced MASH and fibrosis, marking them as essential pharmacological targets for treatment [30].

Triazole-containing heterocyclic scaffolds are uniquely positioned in the medicinal chemistry field due to their impressive versatility as pharmacophores. These molecules have demonstrated broad-spectrum biological activities, including anti-inflammatory, antioxidant, antifungal, antidiabetic, and hepatoprotective effects, making them compelling options for drug discovery targeting MASLD [31]. The 1,2,3- and 1,2,4-triazole rings confer advantageous drug-like properties, including improved metabolic stability, hydrogen-bond acceptor/donor capability, and the ability to engage diverse biological targets via dipole-dipole interactions and π -stacking [32, 33]. Building on the advantageous drug-like properties of triazole scaffolds, our group recently reported the design, synthesis, and complete chemical characterization of a novel triazole derivative, designated as **BTA** [34]. In the current study, we present the comprehensive *in vitro* biological evaluation and mechanistic profiling of this compound (hereafter referred to as **molecule 3**) utilizing a well-established palmitic acid (PA)-induced lipotoxicity model in human hepatocellular carcinoma cells (HepG2). Our experimental design integrates multiple methodologies, including cell viability profiling, fluorescent lipid quantification (BODIPY), oxidative stress assessment (H₂DCFDA), mitochondrial health monitoring (Mito Tracker, JC-1), apoptosis characterization (Annexin V/PI), and broad RT-qPCR gene expression analysis encompassing lipogenic, lipolytic, bile acid biosynthetic, and pro-inflammatory pathways. This study elucidates the molecular mechanisms underlying molecule 3 hepatoprotective activity. Collectively, this study provides mechanistic insight into the biological activity of molecule **3** and supports its further evaluation in advanced preclinical models of MASLD.

2. Materials and methods

2.1. Cell culture

HepG2 cells were received from the NCCS in Pune. Dulbecco's Modified Eagle Medium (DMEM, Sigma # D6546) containing 4.5 g/L glucose, 10% foetal bovine serum (FBS), 1% Penicillin-Streptomycin solution (containing 10,000 units/ml penicillin and 10,000 µg/ml streptomycin), and 0.1% L-glutamine (200 mM). A humidified environment with 5% CO₂ and 95% relative humidity was used to incubate cell cultures at 37°C. Up to fifteen passes of the cultures were preserved through subculturing [29, 35].

2.2. MTT assay

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) test (Hi media, India; Cat. # RM1131) was used to measure cell viability. HepG2 cells were plated at a density of 5×10³ cells per well in 96-well plates and allowed to adhere overnight. The cells were then incubated for 24 hours at 37°C in a 5% CO₂ atmosphere after exposure to 250 µM Palmitic acid (SRL Chemical, India; Cat. # 34262), either alone or in combination with molecule **3**. Following treatment, the medium was aspirated, and a 0.5 mg /ml MTT solution prepared in serum-free media was added to each well. After that, the plates were incubated at 37°C for four hours. After incubation, the insoluble formazan crystals were dissolved in DMSO, and the MTT solution was discarded. As previously mentioned, absorbance measurements were made at 570 nm using a microplate reader [29, 35].

2.3. Experimental Groups

Throughout the study, cells were divided into three experimental groups: Control group [1] HepG2 cells treated with vehicle (0.1% DMSO in complete DMEM); PA group [2] HepG2 cells exposed to 250 µM PA for 24 hours; and PA + molecule **3** group [3] HepG2 cells treated with 250 µM PA for 24 hours after which the media was supplemented with [125 µM] molecule **3** for an additional 4 hours. The treatment duration was selected based on preliminary optimisation experiments and published protocols for PA-induced lipotoxicity.

2.4. BODIPY 493/503 Fluorescent Lipid Staining

HepG2 cells were first cultivated (1×10⁵ cells/well) on a 6-well plate under standard conditions for the BODIPY test. The following day, the cells were treated with molecule **3** and PA to induce lipid formation, and then incubated for a full day. After removing the remaining medium with two DPBS washes, cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature. After fixation, the cells underwent another PBS wash and a 30-minute incubation with a 2 µM BODIPY labelling solution. Following staining, the cells were washed to remove excess dye, and DAPI was used to counterstain the nuclei. The dyed cells were then photographed using an Olympus FV1000SPD confocal microscope. Images were examined for fluorescence intensity using ImageJ (v1.53) for quantification [36].

2.5. Reactive oxygen species detection: H2DCFDA assay

The H2DCFDA fluorescent probe was used to assess intracellular ROS levels according to a modified version of a published procedure [37]. HepG2 cells were first grown (1×10⁵ cells / well) on a 6-well plate under standard conditions for the H2DCFDA test. After that, cells were exposed to the experimental substances for the specified time. After that, cells were exposed to 10 µM H2DCFDA (Cat# C6827,

Invitrogen, USA) for 45 minutes in the dark at 37°C. The cells were then fixed with 4% paraformaldehyde for 10 minutes after being washed at least twice with DPBS to remove excess H2DCFDA. Lastly, a confocal microscope (Olympus FV1000SPD) was used to obtain images. Images were examined for fluorescence intensity using ImageJ (v1.53) for quantification.

2.6. Mito Tracker assay for mitochondrial viability

Using the Mito Tracker test, viable mitochondria in the cell samples were evaluated using the protocol outlined by Ahmed et al. In this process, cells were treated for 45 minutes at 37°C with 400 nM Mito Tracker™ Red CMXRos Dye (Invitrogen, Cat # M46752, USA). The remaining processes are identical to those of H2DCFDA [37, 38].

2.7. Mitochondrial Membrane Potential: JC-1 Assay by Flow Cytometry

The JC-1 assay was performed according to a previously published procedure [38, 39]. In short, HepG2 cells were first seeded in 6-well plates at the proper density (1 million /ml) and left to adhere in full DMEM overnight. After that, experimental substances were applied to the cells. Upon treatment, the cells were gently washed with DPBS, and the culture media was aspirated. After preparing JC-1 dye (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) at a concentration of 3 µM in staining buffer, it was applied to the cells and incubated for 30 minutes at 37°C (Cat #420200, Sigma Aldrich, US). Following incubation, the cells were resuspended in a new solution after being twice washed with DPBS buffer to get rid of extra dye. Using a CytoFLEX™ flow cytometer (Beckman Coulter, USA), the cell suspension was examined for green (monomeric JC-1) and red (J-aggregates). Red/green fluorescence was measured using Cyt Expert SRT software (Beckman Coulter, USA) to determine mitochondrial membrane potential ($\Delta\psi_m$); a reduction in this ratio indicates potential loss.

2.8. Apoptosis Analysis: Annexin V/PI Flow Cytometry

Annexin V/propidium iodide (PI) staining assay and flow cytometry were used to assess apoptosis in the HepG2 cells. HepG2 cells were grown under normal conditions in complete Dulbecco Modified Eagle Medium (DMEM). Following experimental procedures, cells were trypsinized and centrifuged at 3000 rpm for 3 min. A pellet of cells was then washed twice in cold Dulbecco's phosphate-buffered saline (DPBS) and resuspended in 1× binding buffer containing 1×10^6 cells/ml. To stain, 100 µl of cell suspension was left in contact with 5 µl of the solution of fluorochrome-conjugated Annexin V and 5 µl of propidium iodide (PI) solution (Elabscience, USA; Cat. No. E-CK-A211). The suspension was swirled and allowed to rest at room temperature (10–15 minutes) in the dark. After incubation, the samples were diluted in binding buffer and measured with a CytoFLEX flow cytometer (Beckman Coulter, USA). Not less than 10,000 events were collected per sample, and the cell populations were defined as viable, early apoptotic, late apoptotic, and necrotic based on Annexin V and PI staining patterns [39].

2.9. RNA Isolation and Quantitative Gene Expression Analysis by RT-qPCR

RNA Isolation, cDNA Synthesis and Quantitative Real-Time PCR Analysis. TRIzol reagent (Thermo Fisher Scientific, USA) was used to extract total RNA from HepG2 cells according to the manufacturer's instructions. The Nano Drop One spectrophotometer was used to determine the RNA concentration and purity, and the samples with an A260/A280 ratio of greater than 1.8 were considered acceptable, and agarose gel electrophoresis was also used to verify integrity. Prime Script RT Reagent Kit (Takara Bio, Japan) was used to synthesize complementary DNA (cDNA) of 1 µg of total RNA in the presence of random hexamers. Real-time PCR was performed quantitatively using SYBR Green Master Mix (Thermo Fisher Scientific, USA) on a Quant Studio™ 7 Flex Real-Time PCR System (Applied Biosystems, USA). Each reaction (10 µl) was composed of 5 µl of master mix, 0.5 µl of forward and reverse primers (300 nM final concentration), 2 µl of cDNA template, and nuclease-free water. Thermal cycling conditions were as follows: an initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 15s and 60°C for 1min. The result was a melting curve analysis to confirm amplification specificity. The comparative ($2^{-\Delta\Delta C_t}$) method was used to calculate relative gene expression levels using both GAPDH and β -actin as internal reference genes. Target genes (SREBP-1c, ChREBP, ACC, SHP, PPAR- α , SCD1, CD36, CYP7A1, TNF- α , IL- 6, IL- 12, and IFN- γ) primer sequences were generated by Primer-BLAST (NCBI) and purchased from Eurofins, Genomics Pvt Ltd and the primer sequence is attached in the supplementary file Table 1. Experiments were done in biological triplicate [40].

2.10. Statistical Analysis

Data information is given in mean and standard error of the mean (SEM). The statistical tests were conducted using appropriate methods based on the number of groups being compared. A Student t-test was used to compare two groups, and a one-way analysis of variance (ANOVA) was used to compare more than two groups, with multiple comparisons performed using the Tukey post hoc test. A p-value < 0.05 was taken as significant. Origin 2026 software was utilised for all statistical calculations.

3. Results

3.1. Synthesis of molecule 3

The synthetic route for molecule **3**, a 1,2,3-triazole derivative, is illustrated in Fig. 1. The precursor molecule **1** was first converted to the corresponding azide intermediate molecule **2** by reaction with sodium azide and sodium nitrite in trifluoroacetic acid/water, affording the product in high yield. molecule **3** was subsequently obtained via copper(I)-catalysed azide-alkyne cycloaddition (CuAAC), a click reaction, between molecule **2** and phenylacetylene, using CuI and DIPEA as catalysts in DMF at 25°C. The compound has been fully characterised as previously described [34].

3.2. Cytotoxicity Profiling and Selection of Therapeutic Dose Window

The cytotoxicity of molecule **3** was first evaluated across a broad concentration range (0-400 μ M) in HepG2 cells using the MTT assay (Fig. 2A). The molecule **3** exhibited a concentration-dependent reduction in cell viability at higher doses; however, cellular viability remained above 87% at 125 μ M, a concentration identified as safe for pharmacological investigation. Based on this evidence, 125 μ M concentration was therefore selected for subsequent mechanistic investigations. Vehicle control cells treated with 0.1% DMSO showed no significant reduction in viability, confirming solvent-independent toxicity. The concentration of 250 μ M PA was selected to induce lipotoxicity in HepG2 cells, as it reliably induces lipid overaccumulation, mitochondrial dysfunction, apoptosis, and pro-inflammatory gene expression, in accordance with the established literature [36, 41]. Cell viability following co-treatment with 250 μ M PA and 125 μ M molecule **3** is shown in Fig. 2B.

3.3. The molecule **3** Attenuates PA-Induced Intracellular Lipid Accumulation

To recapitulate the hallmark steatotic phenotype of MASLD, HepG2 cells were exposed to 250 μ M PA for 24 hours and stained with BODIPY (Boron-dipyrromethene) 493/503, a fluorescent dye selective for neutral lipids in intracellular lipid droplets. PA-treated cells [case 2 in Fig. 3A] exhibited a pronounced increase in green fluorescence relative to vehicle controls [case 1, Fig. 3A] (**** $p < 0.0001$), with confocal micrographs revealing abundant, large, and widely dispersed lipid droplets throughout the cytoplasm, consistent with lipotoxicity-driven triglyceride (TG) accumulation [42]. Co-treatment with 125 μ M molecule **3** [case 3, Fig. 3A] markedly reduced both the number and size of intracellular lipid droplets. Quantitative image analysis using ImageJ software demonstrated that lipid accumulation was reduced by 65.6% relative to the PA-only group [case 3 vs. case 2, Fig. 3B] (#### $p < 0.0001$). The reduction in intracellular lipid accumulation suggests attenuation of the steatotic phenotype induced by PA and is consistent with the subsequent modulation of genes involved in hepatic lipid synthesis and metabolic homeostasis.

3.4. The molecule **3** Reduces PA-Induced Reactive Oxygen Species Generation

Given the established mechanistic link between hepatic lipid overaccumulation and oxidative stress in MASLD progression, intracellular ROS levels were assessed using the H2DCFDA fluorescent probe. Exposure to 250 μ M PA for 24 hours resulted in a significant elevation of DCF fluorescence intensity in PA-treated cells [case 2, Fig. 4A] compared to controls [case 1, Fig. 4A], confirming robust ROS induction under lipotoxic conditions [43]. Co-treatment with molecule **3** [case 3, Fig. 4A] substantially attenuated this oxidative response, with quantitative ImageJ analysis revealing a 52.8% reduction in DCF fluorescence intensity relative to the PA group [case 3 vs. case 2, Fig. 4B] (#### $p < 0.0001$). The residual fluorescence in molecule **3**-treated cells approached levels observed in vehicle controls [case 1, Fig. 4B], indicating effective restoration of cellular redox homeostasis under lipotoxic challenge. Given the central role of oxidative stress in the progression of lipotoxic liver injury, the observed reduction in ROS levels suggests that molecule **3** mitigates a key pathological driver of MASLD progression. Representative

confocal micrographs further confirmed the marked reduction in intracellular ROS-associated fluorescence following treatment with molecule **3**.

3.5. The molecule **3** Preserves Mitochondrial Integrity Under Lipotoxic Conditions

Mitochondrial viability was evaluated using MitoTracker Red CMXRos, a membrane potential-dependent fluorescent dye that selectively accumulates in metabolically active mitochondria and serves as a quantitative indicator of mitochondrial mass and functional integrity. PA exposure at 250 μ M caused a significant reduction in Mito Tracker Red fluorescence intensity, declining from 2.24 in control cells [case 1, Fig. 5B] to 1.01 in PA-treated cells [case 2, Fig. 5B] (**** $p < 0.0001$), consistent with PA-induced impairment of mitochondrial mass and activity reported in prior studies [44]. Co-treatment with molecule **3** significantly recovered fluorescence intensity to 1.93 [case 3, Fig. 5B] (## $p < 0.01$ vs. PA group), demonstrating effective preservation of mitochondrial structural integrity under lipotoxic conditions.

3.6. The effect of molecule **3** Restores Mitochondrial Membrane Potential ($\Delta\Psi_m$)

The functional consequence of PA-induced mitochondrial impairment on membrane potential ($\Delta\Psi_m$) was quantified by flow cytometry using JC-1 staining. In healthy, energetically coupled mitochondria, JC-1 forms red-fluorescent J-aggregates, whereas in depolarised mitochondria it remains as green-fluorescent monomers. In control cells [case 1, Fig. 6A and 6B], the red/green JC-1 fluorescence ratio was 1.99, indicative of intact $\Delta\Psi_m$. PA treatment at 250 μ M caused a pronounced shift from J-aggregates to JC-1 monomers, with the red/green ratio dropping to 0.52 [case 2, Fig. 6A and 6B] (**** $p < 0.0001$), confirming severe mitochondrial depolarisation, in agreement with established reports that palmitic acid disrupts mitochondrial energetics in hepatocytes [45]. Co-treatment with molecule **3** effectively restored $\Delta\Psi_m$, with the red/green ratio recovering to 1.71 [case 3, Fig. 6A and 6B] relative to 0.52 in the PA-only group [case 2, Fig. 6A and 6B] (#### $p < 0.0001$), demonstrating substantial restoration of PA-induced mitochondrial depolarisation.

3.7. The molecule **3** significantly reduced apoptotic cell death

Apoptosis was quantified by Annexin V-FITC/Propidium Iodide (PI) double staining followed by flow cytometric analysis (Fig. 7). In vehicle-treated control cells [case 1, Fig. 7A and 7B], the viable (Annexin V⁻/PI⁻) population predominated, with a total apoptotic fraction of 1.56%. PA exposure at 250 μ M markedly increased both early (Annexin V⁺/PI⁻) and late (Annexin V⁺/PI⁺) apoptotic populations, with total Annexin V-positive cells reaching 55.23% [case 2, Fig. 7A and 7B] (**** $p < 0.0001$ vs. control), consistent with the pro-apoptotic consequences of saturated fatty acid-induced lipotoxicity [46–48]. Co-treatment with molecule **3** resulted in a significant redistribution of cells towards the viable fraction, with

the total Annexin V-positive population declining to 26.80% [case 3, Fig. 7A and 7B] (#### $p < 0.0001$ vs. PA group), representing a substantial reduction in apoptotic cell death under lipotoxic conditions.

3.8. Effect of molecule 3 on Lipogenic and Metabolic Gene Expression

To elucidate the transcriptional basis of the anti-steatotic phenotype observed in lipid accumulation assays, RT-qPCR analysis was performed for a panel of genes governing hepatic lipid homeostasis (Fig. 8). PA treatment markedly increased CD36 expression, a major fatty acid translocase [case 2, Fig. 8E], reflecting enhanced lipid influx into hepatocytes [49]. Concurrently, the lipogenic transcription factors SREBP-1c and ChREBP [case 2, Fig. 8A and 8B], together with their downstream effectors ACC and SCD1 [case 2, Fig. 8C and 8D], were significantly upregulated, indicating activation of the *de novo* lipogenic programme [50, 51]. Conversely, PA exposure led to a significant downregulation of PPAR α [case 2, Fig. 8F], a key transcriptional activator of mitochondrial fatty acid β -oxidation, alongside SHP [case 2, Fig. 8G], a nuclear receptor that restrains lipogenic gene expression. CYP7A1 expression [case 2, Fig. 8H] was also reduced, consistent with impaired cholesterol catabolism and bile acid biosynthesis [52, 53].

Co-treatment with molecule 3 [case 3, Fig. 8A-8H] reversed these PA-induced transcriptional alterations across all targets examined. The expression of SREBP-1c was suppressed by 60.1% [case 3, Fig. 8A], ChREBP by 60.8% [case 3, Fig. 8B], ACC by 71.1% [case 3, Fig. 8C], and SCD1 by 87.4% [case 3, Fig. 8D] (#### $p < 0.0001$ vs. PA group), indicating potent inhibition of *de novo* lipogenesis. CD36 expression was downregulated by 82.1% [case 3, Fig. 8E] (#### $p < 0.0001$), reflecting reduced fatty acid uptake. In parallel, molecule 3 substantially restored PPAR α expression by 312.9% (4.1-fold) [case 3, Fig. 8F] and SHP by 102.4% [case 3, Fig. 8G] (#### $p < 0.0001$), thereby enhancing fatty acid β -oxidation capacity and re-establishing negative regulation of lipogenic transcription. CYP7A1 expression was recovered by 40.3% [case 3, Fig. 8H] (#### $p < 0.0001$), indicating improved cholesterol metabolic flux and bile acid homeostasis.

3.9. The molecule 3 Suppresses PA-Induced Transcriptional Upregulation of Pro-Inflammatory Cytokines

The effect of molecule 3 on hepatic inflammatory signalling was assessed by RT-qPCR quantification of the key pro-inflammatory cytokines TNF- α , IL-6, IL-12, and IFN- γ in PA-exposed HepG2 cells (Fig. 9). PA treatment significantly upregulated the mRNA expression of all four cytokines relative to vehicle controls [case 1, Fig. 9A–9D] (**** $p < 0.0001$), consistent with the established pro-inflammatory consequences of lipotoxic injury in hepatocytes [37]. Co-treatment with molecule 3 [case 3, Fig. 9A–9D] significantly attenuated this transcriptional upregulation, reducing TNF- α by 60.3%, IL-6 by 72.1%, IL-12 by 72.1%, and IFN- γ by 87.2% (#### $p < 0.0001$ vs PA group).

4. Discussion

The present study provides the first comprehensive *in vitro* mechanistic evaluation of molecule **3**, a novel 1,2,3-triazole derivative, in a palmitic acid (PA)-induced lipotoxicity model in HepG2 cells. Employing an integrated multi-endpoint experimental strategy, our data demonstrate that molecule **3** simultaneously attenuates the principal pathological hallmarks of MASLD at the cellular level, hepatic steatosis, oxidative stress, mitochondrial dysfunction, hepatocellular apoptosis, and pro-inflammatory cytokine upregulation, while concurrently restoring the transcriptional programme governing hepatic lipid homeostasis. Taken together, these findings indicate that molecule **3** modulates multiple pathological processes associated with PA-induced lipotoxicity and provide a mechanistic basis for its further evaluation in MASLD-relevant models.

The defining and initiating pathological event in MASLD is the excessive accumulation of lipid species within hepatocytes, which subsequently drives a cascade of downstream injury mechanisms [42]. In the present study, PA-treated HepG2 cells exhibited a dramatic increase in intracellular neutral lipid content, confirmed by BODIPY 493/503 fluorescence, with confocal microscopy revealing large, widely dispersed lipid droplets consistent with lipotoxicity-induced TG accumulation (Fig. 3A). Co-treatment with molecule **3** at 125 μ M markedly reduced both the number and size of lipid droplets, with quantitative analysis confirming a 65.6% reduction in lipid accumulation relative to the PA group (Fig. 3B, ##### $p < 0.0001$). Because intracellular lipid overaccumulation serves as a major source of lipotoxic stress, the reduction in steatosis observed following molecule **3** treatment may contribute to the subsequent attenuation of oxidative stress and mitochondrial dysfunction. The transcriptional data from Fig. 9 provide a mechanistic basis for this phenotypic outcome: molecule **3** potently suppressed the lipogenic transcription factors SREBP-1c and ChREBP alongside their downstream effectors ACC and SCD1, and simultaneously reduced CD36-mediated fatty acid uptake, indicating coordinated inhibition of the *de novo* lipogenic programme and exogenous fatty acid influx [50]. The overaccumulation of lipid species within hepatocytes is mechanistically coupled to elevated ROS production, a critical driver of the transition from simple steatosis to more advanced inflammatory and apoptotic stages of MASLD. As demonstrated in Fig. 4A and 4B, PA exposure induced a robust increase in intracellular ROS, confirmed by H2DCFDA fluorescence; co-treatment with molecule **3** significantly attenuated this oxidative burden, reducing DCF fluorescence intensity by 52.8% (##### $p < 0.0001$), with residual fluorescence approaching control levels. These findings indicate that molecule **3** suggesting antioxidant potential and/or enhances endogenous antioxidant defence mechanisms in hepatocytes under lipotoxic stress, consistent with the known capacity of 1,2,3-triazole scaffolds to engage diverse antioxidant targets through their hydrogen-bond acceptor and donor functionalities [32]. Mitochondrial integrity represents a critical nexus in MASLD pathophysiology, as excessive ROS production is a principal driver of mitochondrial membrane damage and bioenergetic deterioration. The observed reduction in ROS is particularly relevant because oxidative stress is a key mediator linking hepatic steatosis to mitochondrial dysfunction and apoptotic signalling during MASLD progression. demonstrating near-complete reversal of $\Delta\Psi_m$ loss. Assessment of mitochondrial viability using Mito Tracker Red CMXRos revealed a significant decline in fluorescence intensity from 2.24 in controls to 1.01 in PA-exposed cells (Fig. 5B, **** $p < 0.0001$), reflecting loss of

mitochondrial mass and activity consistent with prior reports of PA-induced mitochondrial impairment [44]. Co-treatment with molecule **3** restored mitochondrial fluorescence to 1.93 (Fig. 5B, $##p < 0.01$ vs. PA group). This structural preservation was corroborated by JC-1 flow cytometry, which demonstrated that the red/green fluorescence ratio dropped from 1.99 in controls to 0.52 in PA-treated cells (Fig. 6B, $****p < 0.0001$), confirming severe mitochondrial depolarisation, a hallmark of PA-induced mitochondrial dysfunction in hepatocytes [54]. Crucially, molecule **3** co-treatment restored the red/green ratio to 1.71 (Fig. 6B, $####p < 0.0001$), indicating substantial restoration of mitochondrial membrane potential. This protective effect on mitochondrial membrane potential is mechanistically consistent with the antioxidant activity described above, as ROS-mediated oxidative damage is a primary initiator of the mitochondrial permeability transition in lipotoxic hepatocytes.

The mechanistic continuity between mitochondrial depolarisation and downstream apoptotic commitment is well established, with loss of $\Delta\Psi_m$ representing an early and pivotal event in the intrinsic apoptotic pathway through mitochondrial outer membrane permeabilisation (MOMP). This mechanistic link is supported by the apoptosis data presented in Fig. 7: PA exposure increased the total Annexin V-positive population from 1.56% in controls to 55.23% ($****p < 0.0001$), reflecting extensive activation of apoptotic programmes under lipotoxic conditions [46–48]. Co-treatment with molecule **3** markedly reduced this apoptotic fraction to 26.80% (Fig. 7B, $####p < 0.0001$), with a clear redistribution towards the viable population. The sequential relationship between the recovery of $\Delta\Psi_m$ observed in Fig. 6B and this downstream reduction in apoptotic cell death strongly supports the interpretation that molecule **3** interrupts the intrinsic apoptotic cascade at the level of mitochondrial depolarisation, prior to the irreversible commitment to cell death.

Hepatic inflammation is a crucial factor driving the progression of MASLD towards MASH and fibrosis [2]. The RT-qPCR data presented in Fig. 9 demonstrate that PA treatment significantly elevated the mRNA expression of TNF- α , IL-6, IL-12, and IFN- γ in HepG2 cells, consistent with lipotoxicity-driven hepatic inflammatory signalling [44]. Co-treatment with molecule **3** potently suppressed this transcriptional upregulation, reducing TNF- α by 60.3%, IL-6 by 72.1%, IL-12 by 72.1%, and IFN- γ by 87.2% (Fig. 9A–9D, $####p < 0.0001$). The particularly pronounced suppression of IFN- γ is noteworthy, given the recently characterised IFN- γ /IL-12 axis as a key regulator of intercellular crosstalk in MASLD progression [55]. This broad anti-inflammatory activity, combined with the antioxidant and anti-apoptotic effects described above, suggests that molecule **3** may act upstream of canonical inflammatory signalling. The broad reduction in cytokine transcript expression suggests that molecule **3** may influence upstream pathways involved in coordinating metabolic and inflammatory responses during lipotoxic stress; however, the precise signalling mechanisms require further investigation. The transcriptional data presented in Fig. 8 offer a comprehensive molecular framework for the hepatoprotective phenotype observed across all functional assays. Hepatic lipid balance is governed by the dynamic interplay between lipid acquisition, principally *de novo* lipogenesis, and exogenous fatty acid uptake, and lipid disposal through mitochondrial fatty acid β -oxidation and cholesterol/bile acid catabolism [56]. PA treatment disrupted this balance at multiple nodes: CD36 expression was elevated (Fig. 8E), indicating enhanced fatty acid influx; SREBP-1c, ChREBP, ACC, and SCD1 were markedly upregulated (Fig. 8A–8D), reflecting activation

of the *de novo* lipogenic programme [50, 51]. PPAR α and SHP were downregulated (Fig. 8F and 8G), indicating suppressed fatty acid oxidation and impaired feedback restraint of lipogenesis; and CYP7A1 was reduced (Fig. 8H), suggesting impaired cholesterol catabolism and bile acid synthesis [52, 53]. The molecule **3** reversed all of these alterations in a consistent and statistically significant manner. The suppression of lipogenic gene expression (SREBP-1c by 60.1%, ChREBP by 60.8%, ACC by 71.1%, SCD1 by 87.4%), reduction in CD36-mediated fatty acid uptake (82.1%), restoration of PPAR α (4.1-fold) and SHP (102.4%), and recovery of CYP7A1 (40.3%) collectively indicate that molecule **3** orchestrates a coordinated metabolic reprogramming that simultaneously curtails lipid acquisition and promotes lipid disposal. These molecular findings are in strong concordance with the phenotypic reductions in lipid accumulation (Fig. 3), oxidative stress (Fig. 4), mitochondrial dysfunction (Figs. 5 and 6), and apoptosis (Fig. 7) described above. Collectively, these findings support the concept that modulation of hepatic lipid metabolism represents a central mechanism underlying the protective effects of molecule **3**, with secondary benefits extending to oxidative stress, mitochondrial function, apoptosis, and inflammatory signalling. Collectively, the transcriptional and functional findings support a unified mechanistic framework for the hepatoprotective effects of molecule **3**. Suppression of CD36-mediated fatty acid uptake and inhibition of the lipogenic regulators SREBP-1c, ChREBP, ACC and SCD1 were associated with reduced intracellular lipid accumulation. Concurrent restoration of PPAR α , SHP and CYP7A1 expression suggests improved fatty acid oxidation and cholesterol homeostasis, which may contribute to the attenuation of oxidative stress, preservation of mitochondrial function, reduction of apoptotic cell death and suppression of pro-inflammatory cytokine expression. The proposed mechanistic model integrating these interconnected pathways is presented in Fig. 10.

Several limitations of the current study merit transparent acknowledgement. First, HepG2 cells, while the most widely employed cellular model for hepatocyte lipid metabolism research, represent a hepatocellular carcinoma-derived line with altered metabolic characteristics relative to primary human hepatocytes; confirmation of these findings in primary hepatocyte cultures or advanced three-dimensional liver organoid systems would substantially strengthen translational relevance. Second, the 24-hour PA treatment window captures acute lipotoxic responses but cannot fully recapitulate the chronic, progressive nature of MASLD; longer-duration experiments and repeated-dosing protocols are warranted in future studies. Third, while RT-qPCR provides robust transcriptional evidence, protein-level confirmation of key regulators, including SREBP-1c, PPAR α , and ACC, by Western blotting, cytokine protein secretion by ELISA, and direct enzymatic activity assays would provide more mechanistically definitive insight. Fourth, the direct molecular target(s) of molecule **3** remain unidentified; future investigations employing molecular docking simulations and thermal proteome profiling are required to establish the precise mechanism of action and to support rational structural optimisation of this scaffold.

5. Conclusion

This study shows that the novel synthetic triazole-based molecule **3** has significant hepatoprotective effects in a palmitate-induced *in vitro* model of metabolic-associated steatotic liver disease (MASLD).

molecule **3** reduces intracellular lipid accumulation, alleviates oxidative stress, preserves mitochondrial integrity, decreases apoptotic cell death, and suppresses pro-inflammatory cytokines. These effects are linked to the downregulation of key genes involved in lipid accumulation (SREBP-1c, ChREBP, ACC, SCD1, CD36) and a restoration of genes promoting fatty acid oxidation (PPAR- α , SHP, CYP7A1). The molecule **3** also significantly reduces pro-inflammatory mediators like TNF- α , IL-6, IL-12, and IFN- γ . Overall, these findings highlight the triazole scaffold as a promising candidate for the treatment of MASLD. Given MASLD's role in the progression to hepatocellular carcinoma (HCC), molecule **3** may have broader implications for slowing disease progression. Further studies are needed for in vivo validation, target identification, and optimization to advance molecule **3** toward clinical application.

Abbreviations

MASLD

Metabolic-dysfunction-associated steatotic liver disease

MASH

Metabolic dysfunction-associated steatohepatitis

HCC

Hepatocellular carcinoma

PA

Palmitic acid

ROS

Reactive oxygen species

FFA

Free fatty acid

TG

Triglyceride

ACC

Acetyl-CoA carboxylase

CD36

Cluster of differentiation 36

ChREBP

Carbohydrate-responsive element-binding protein

CYP7A1

Cytochrome P450 7A1

IFN- γ

Interferon-gamma

IL-6

Interleukin-6

IL-12

Interleukin-12

PPAR α
Peroxisome proliferator-activated receptor-alpha
SCD1
Stearoyl-CoA desaturase 1
SHP
Small heterodimer partner
SREBP-1c
Sterol regulatory element-binding protein-1c
TNF- α
Tumour necrosis factor-alpha
 $\Delta\Psi_m$
Mitochondrial membrane potential
BODIPY
Boron-dipyrromethene (used for fluorescent lipid staining)
DAPI
4',6-diamidino-2-phenylindole
DMEM
Dulbecco's Modified Eagle Medium
DPBS
Dulbecco's phosphate-buffered saline
FBS
Foetal bovine serum
H2DCFDA
2',7'-dichlorodihydrofluorescein diacetate
JC-1
5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide
MMP
Mitochondrial membrane potential assay
MTT
3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
PI
Propidium iodide
RT-qPCR
Reverse transcription-quantitative polymerase chain reaction
SEM
Standard error of the mean

Declarations

8.1. Conflicts of interest

The authors state that they have no identifiable personal or competing conflicts of interest that could have influenced the research presented in this article

8.2. Acknowledgements

The authors would like to express their sincere gratitude to Dr. Sabyasachi Rakshit from the Department of Chemical Sciences at IISER Mohali for generously providing cell culture facilities. We also appreciate his invaluable support in conducting the anti-inflammatory research and the Quantitative Gene Expression Analysis using RT-qPCR.

8.3. Financial assistance

The authors are thankful for the financial assistance from NIPER S.A.S. Nagar, P.S., S.A., and K.M.S. thank NIPER S.A.S. Nagar for their fellowships.

8.4. Ethical approvals

In these studies, there is no involvement of animal or human-related experiments

8.5. Author contributions

PS and SN designed the study and interpreted data; PS, SA, AK, and DS performed the experiments; PS, SA, and SN analysed and visualised the data; SN, DS, and SSS supported the experiment resources; SN supervised the research; PS and KMS wrote the original draft; SN reviewed and edited the paper. All authors agree to be accountable for all aspects of work, ensuring integrity and accuracy.

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Data Availability

Data availabilityThe datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

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Figures

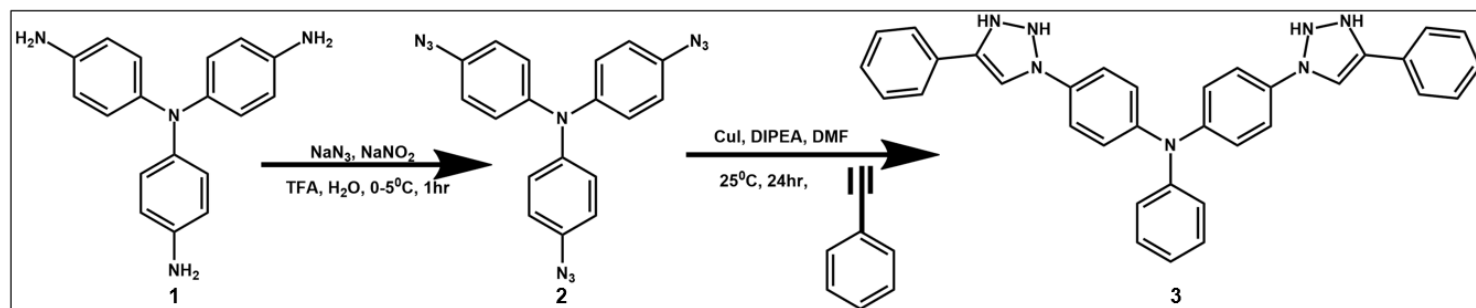


Figure 1

Synthetic route for the triazole-based derivative, molecule 3. Reagents and conditions: (a) NaN₃, NaNO₂, TFA, H₂O; (b) Phenylacetylene, CuI, DIPEA, DMF, 25 °C. The schematic details the progression from precursor (molecule 1) to the azide intermediate (molecule 2), culminating in the final 1,2,3-triazole product (molecule 3).

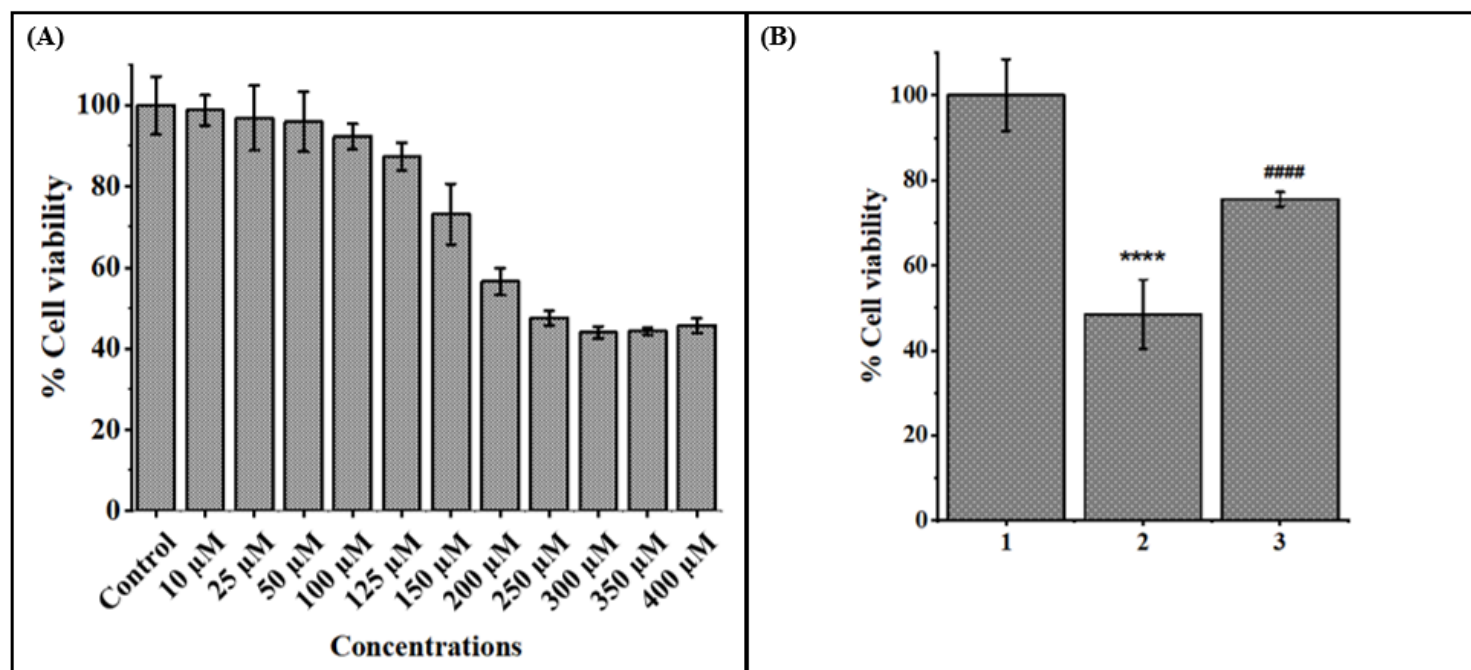


Figure 2

Effect of molecule 3 on cell viability in palmitic acid (PA)-treated HepG2 cells. A: Dose-response effect on cell viability following treatment with increasing concentrations of molecule 3 (10-400 μM) for 24 h, B: Cell viability in HepG2 cells exposed to 250 μM PA for 24 h, followed by a 4-h treatment with 125 μM molecule 3. Data are presented as mean ± SEM of three independent experiments (n=3). Experimental groups: 1: Control cells; 2: PA-treated cells, and 3: PA co-incubated with molecule 3. Statistical significance was determined using One-Way ANOVA followed by Tukey's post hoc test. **** p < 0.0001 compared to the Control group [1]. #### p < 0.0001 compared to the PA-treated group [2].

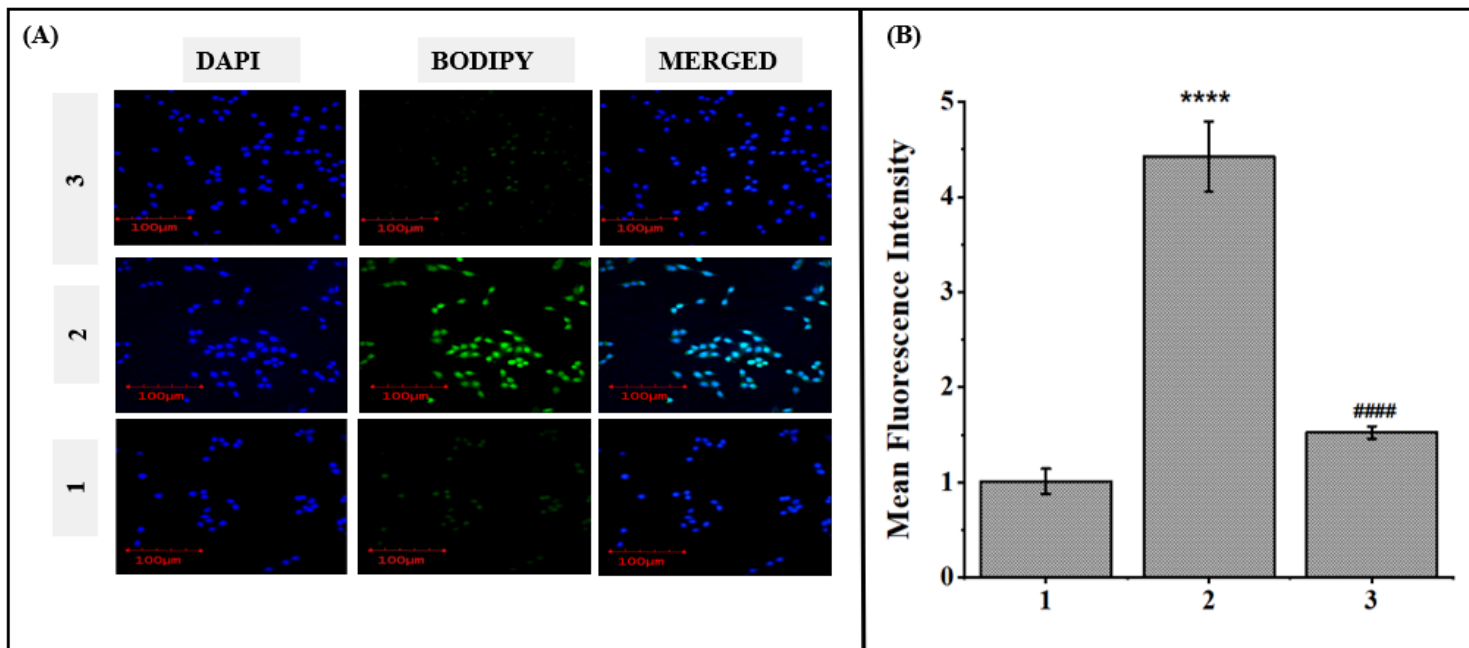


Figure 3

The molecule 3 attenuates PA-induced lipid accumulation in HepG2 cells. A: Representative confocal micrographs of cells stained with BODIPY 493/503 (green, neutral lipids) and DAPI (blue, nuclei). Scale bar = 100 µm, B: Quantitative analysis of mean BODIPY fluorescence intensity, lipid droplet count, and mean lipid droplet area per cell utilising ImageJ software. Data are presented as mean $\pm$ SEM (n=3). Experimental groups: 1: Control cells; 2: PA-treated cells, and 3: PA co-incubated with molecule **3**. Statistical significance was determined using One-Way ANOVA followed by Tukey's post hoc test. **** p < 0.0001 compared to the Control group [1]. #### p < 0.0001 compared to the PA-treated group [2].

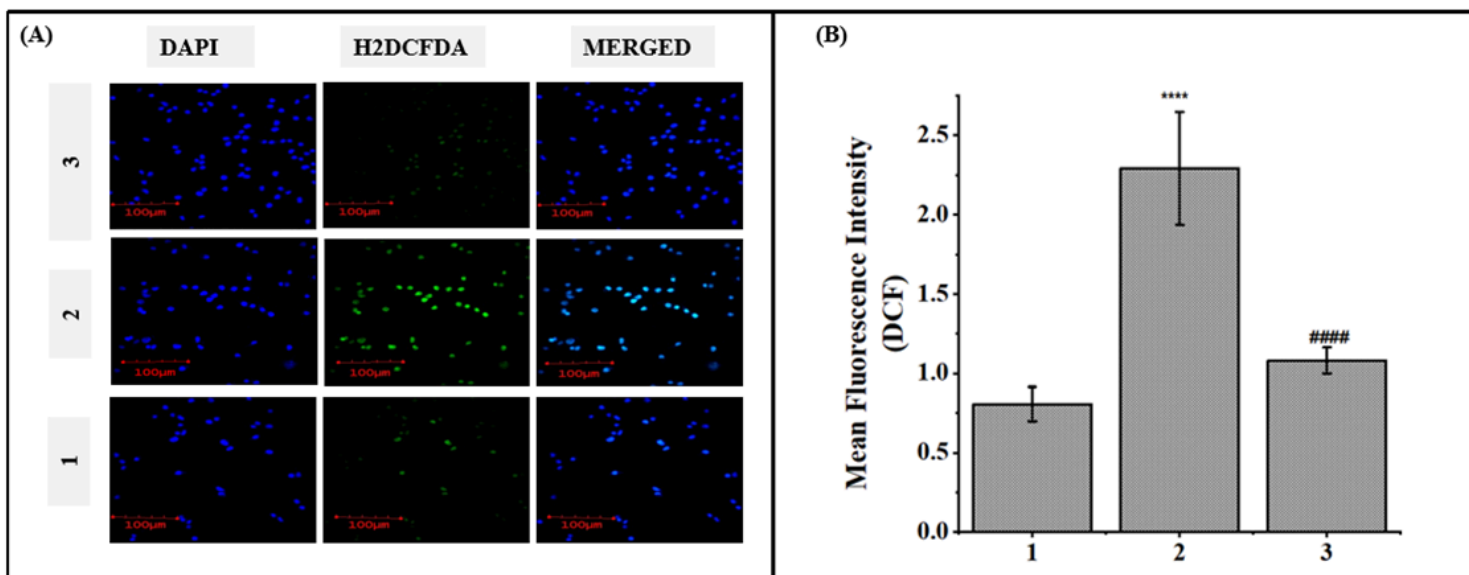


Figure 4

The molecule 3 mitigates PA-induced intracellular reactive oxygen species (ROS) generation. A: Representative confocal fluorescence images of HepG2 cells stained with DCFH-DA (green); B: Quantification of mean DCF fluorescence intensity. Data are presented as mean $\pm$ SEM (n=3). Experimental groups: 1: Control cells; 2: PA-treated cells, and 3: PA co-incubated with molecule **3**. Statistical significance was determined using One-Way ANOVA followed by Tukey's post hoc test. **** p < 0.0001 compared to the Control group [1]. #### p < 0.0001 compared to the PA-treated group [2].

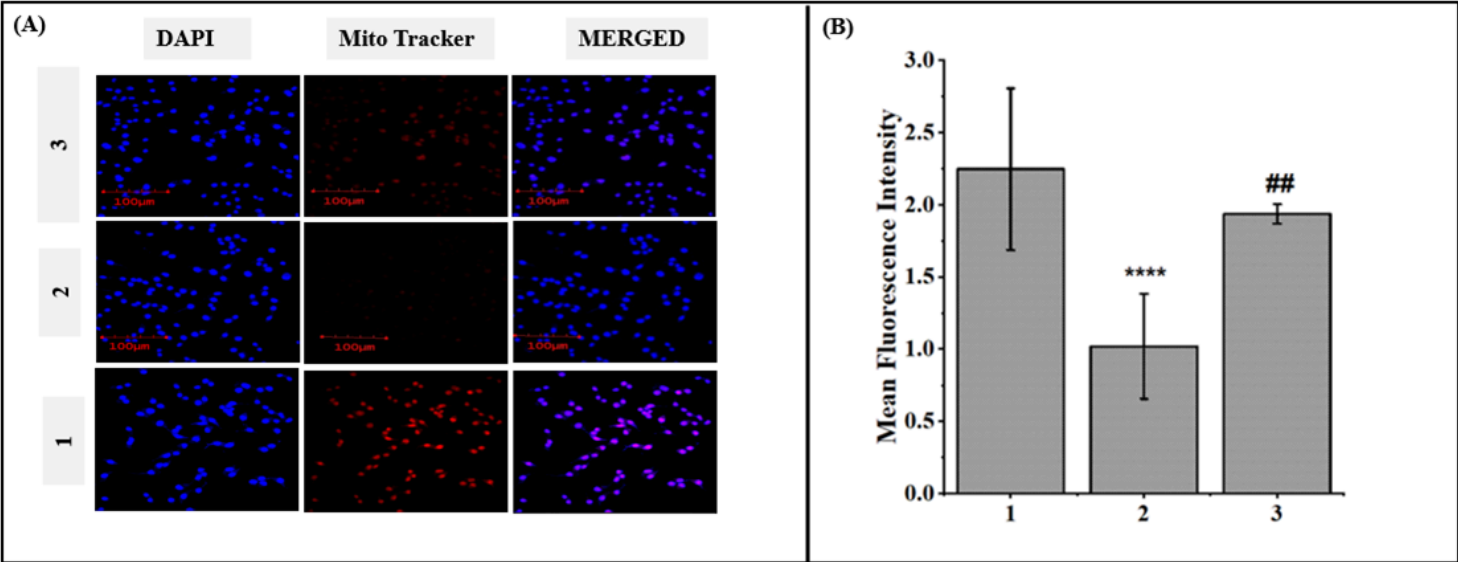


Figure 5

The molecule 3 preserves mitochondrial integrity and morphology under lipotoxic conditions. A: Representative confocal images of HepG2 cells stained with Mito Tracker Red CMXRos to visualise active mitochondria. Scale bar = 100 µm, and B: Quantitative analysis of the mitochondrial integrity index. Data are presented as mean $\pm$ SEM (n=3). Experimental groups: 1: Control cells; 2: PA-treated cells, and 3: PA co-incubated with molecule **3**. Statistical significance was determined using One-Way ANOVA followed by Tukey's post hoc test. ****p < 0.0001 compared to the Control group [1]. ## p < 0.01 compared to the PA-treated group [2].

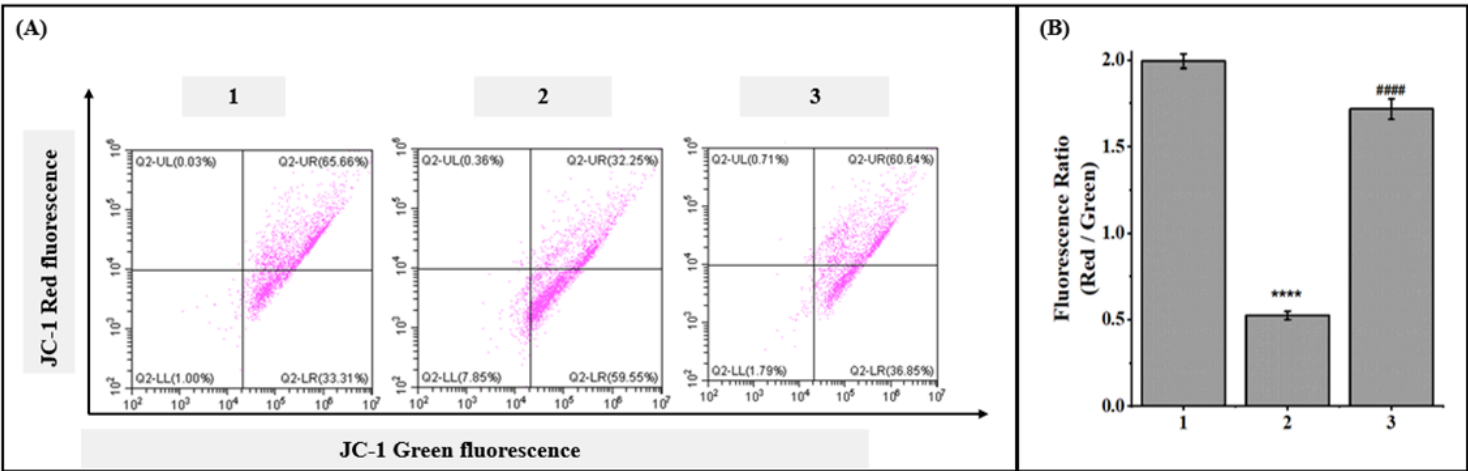


Figure 6

The molecule 3 restores mitochondrial membrane potential ($\Delta\Psi_m$) in PA-treated cells. A:

Representative flow cytometric dot plots depicting JC-1 staining, separating red fluorescence (J-aggregates, healthy polarized mitochondria) and green fluorescence (J-monomers, depolarized mitochondria). B: Quantitative analysis of the red-to-green fluorescence ratio (JC-1 MMP index). Data are presented as mean $\pm$ SEM (n=3). Experimental groups: 1: Control cells; 2: PA-treated cells, and 3: PA co-incubated with molecule **3**. Statistical significance was determined using One-Way ANOVA followed by Tukey's post hoc test. **** p < 0.0001 compared to the Control group [1]. #### p < 0.0001 compared to the PA-treated group [2].

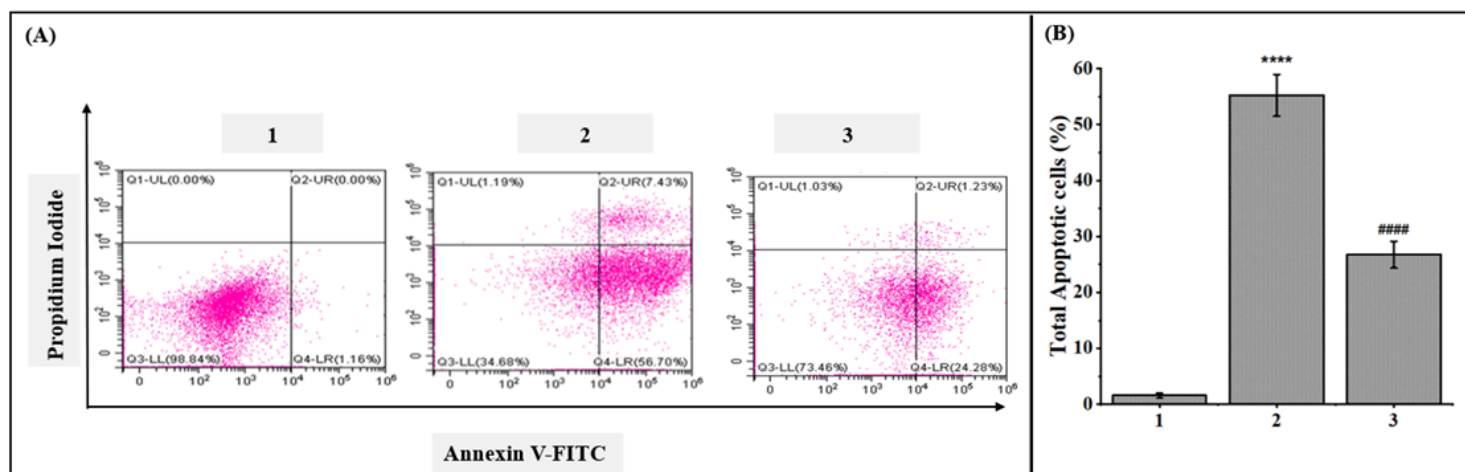


Figure 7

The molecule 3 significantly reduced apoptotic cell death. A: Representative flow cytometric dot plots of HepG2 cells stained with Annexin V-FITC and Propidium Iodide (PI), B: Quantitative bar graph

illustrating the percentages of viable (Annexin V⁻/PI⁻), early apoptotic (Annexin V⁺/PI⁻), late apoptotic (Annexin V⁺/PI⁺), and necrotic cells. Data are presented as mean $\pm$ SEM (n=3). Experimental groups: 1: Control cells; 2: PA-treated cells, and 3: PA co-incubated with molecule **3**. Statistical significance was determined using One-Way ANOVA followed by Tukey's post hoc test. **** p < 0.0001 compared to the Control group [1]. #### p < 0.0001 compared to the PA-treated group [2].

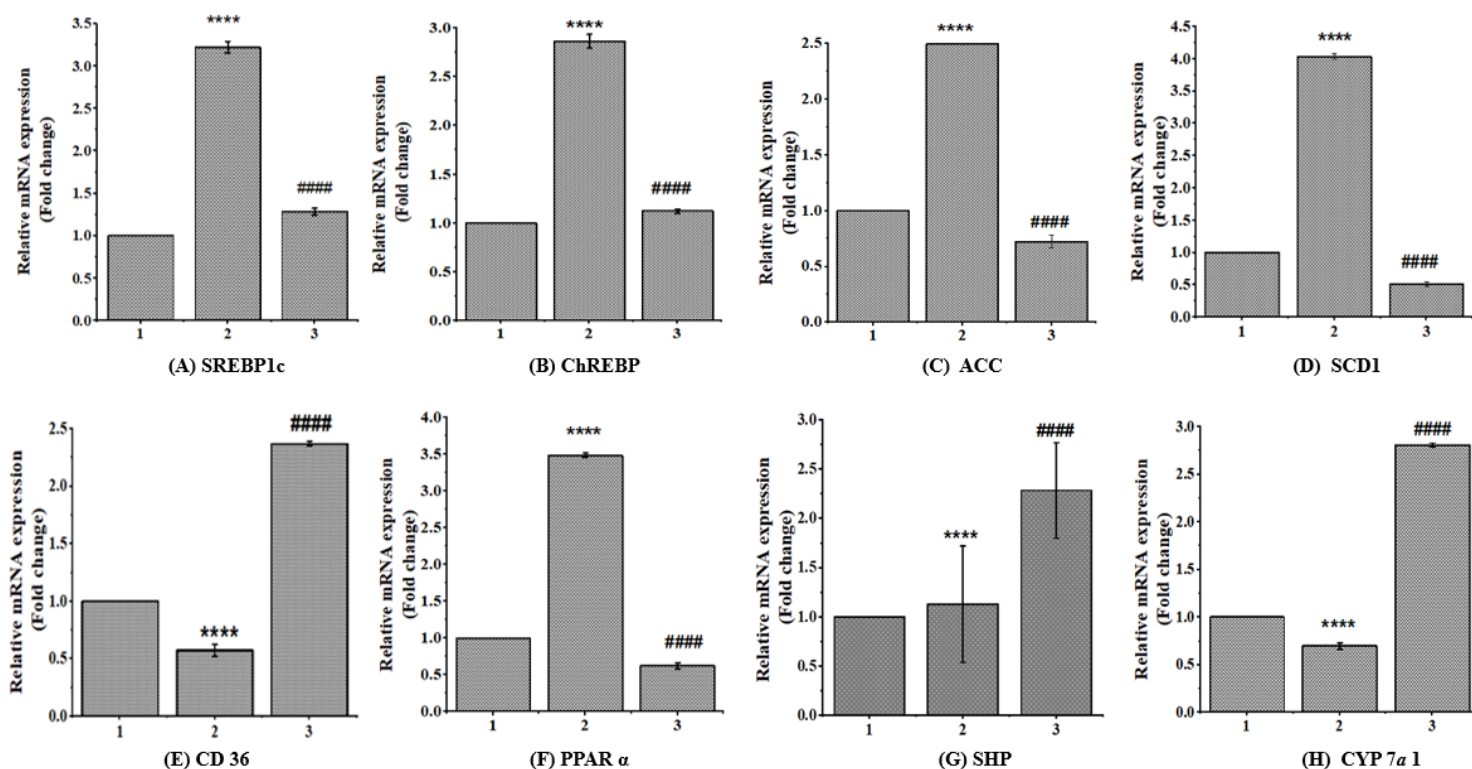


Figure 8

The molecule 3 modulates the expression of lipid metabolism-related genes in PA-treated HepG2 cells.

RT-qPCR analysis of mRNA expression levels for lipogenic and metabolic regulators: (A) SREBP-1c, (B) ChREBP, (C) ACC, (D) SCD1, (E) CD36, (F) PPAR α , (G) SHP, and (H) CYP7A1. HepG2 cells were exposed to 250 μ M PA with or without molecule 3. Relative expression levels were normalised to the GAPDH internal control and calculated using the $2^{-\Delta\Delta C_t}$ method. Data are presented as mean $\pm$ SEM from three independent experiments performed in technical triplicate. Experimental groups: 1: Control cells; 2: PA-treated cells, and 3: PA co-incubated with molecule 3. Statistical significance was determined using One-Way ANOVA followed by Tukey's post hoc test. **** $p < 0.0001$ compared to the Control group [1]. #### $p < 0.0001$ compared to the PA-treated group [2].

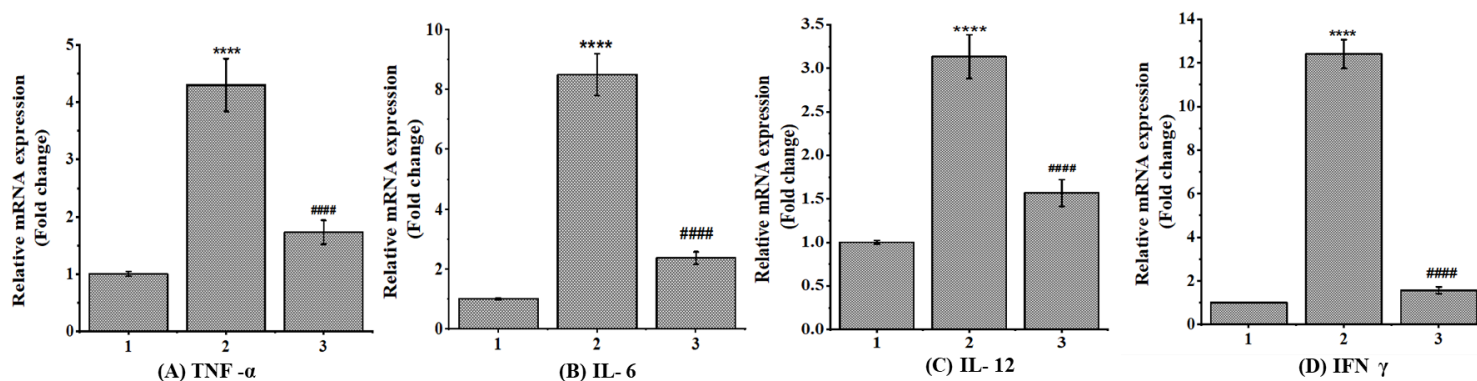


Figure 9

The molecule 3 suppresses PA-induced transcription of pro-inflammatory cytokines. RT-qPCR analysis showing relative mRNA expression levels of (A) TNF- α , (B) IL-6, (C) IL-12, and (D) IFN- γ in HepG2 cells. Data are presented as mean $\pm$ SEM from three independent experiments performed in technical triplicate. Experimental groups: 1: Control cells; 2: PA-treated cells, and 3: PA co-incubated with molecule 3. Statistical significance was determined using One-Way ANOVA followed by Tukey's post hoc test. **** $p < 0.0001$ compared to the Control group [1]. #### $p < 0.0001$ compared to the PA-treated group [2].

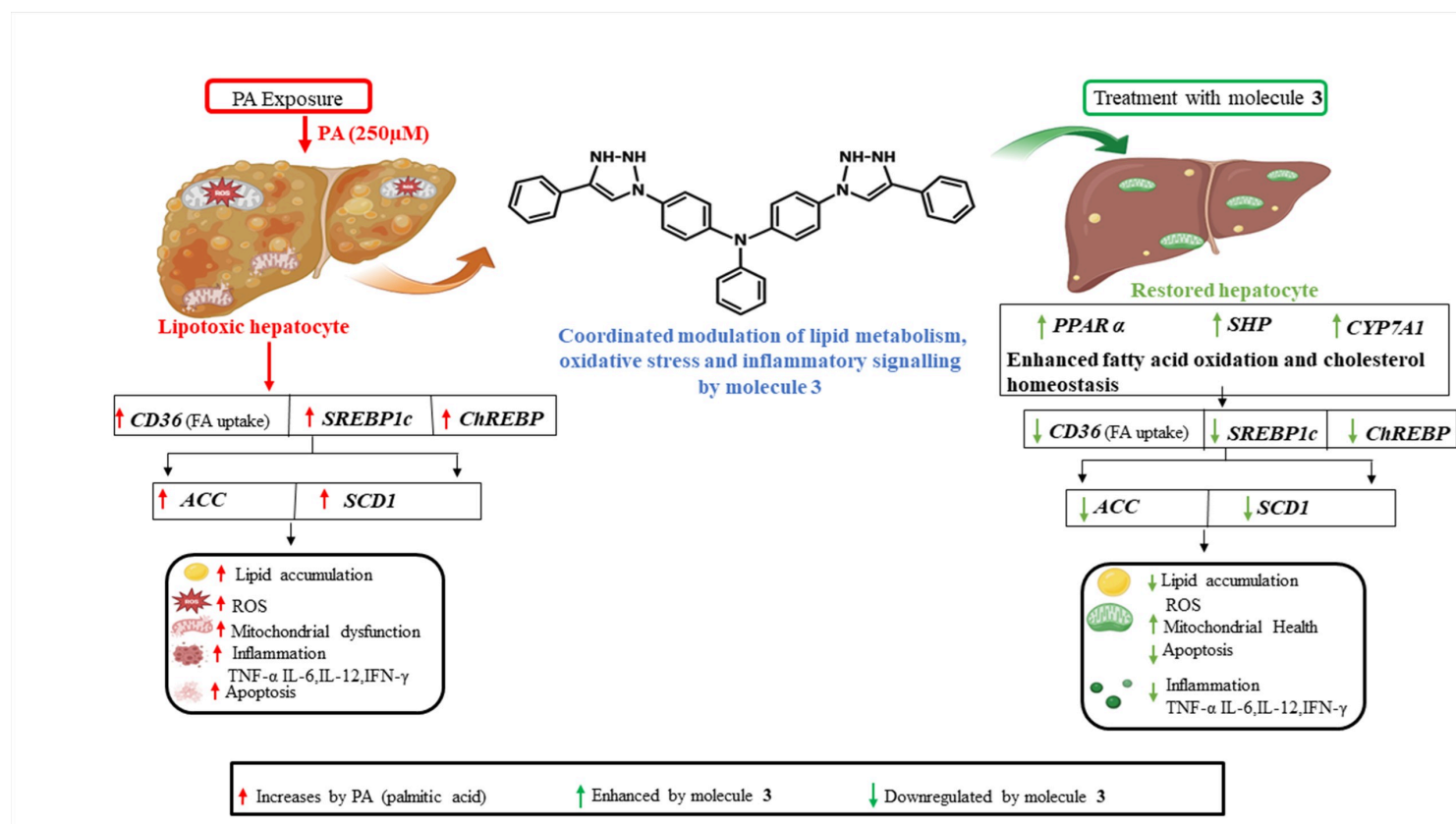


Figure 10

Proposed mechanism underlying the protective effects of molecule 3 against palmitic acid-induced lipotoxicity in HepG2 cells. Palmitic acid (PA) promotes lipotoxicity by increasing fatty acid (FA) uptake and activating lipogenic regulators (*CD36*, *SREBP1c*, *ChREBP*, *ACC*, and *SCD1*), resulting in lipid accumulation, oxidative stress, mitochondrial dysfunction, apoptosis, and inflammatory responses. Molecule 3 suppresses lipogenic signalling while enhancing metabolic regulators (*PPAR α* , *SHP* and *CYP7A1*), thereby promoting fatty acid oxidation and cholesterol homeostasis, reducing lipotoxic injury and restoring hepatocellular function.

Supplementary Files

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