Apoptosis (2019) 24:290–300
https://doi.org/10.1007/s10495-019-01518-y

Protective effect of dihydromyricetin on hyperthermia-induced apoptosis in human myelomonocytic lymphoma cells

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Published online: 25 January 2019

Abstract

Dihydromyricetin (DMY) is a traditional herbal medicine, with a wide range of biological activities. Extreme hyperthermia (HT) can suppress the immune system; thus, protection of the immune system is beneficial in heat-related diseases, including heatstroke. In our study, we revealed the protective effect of DMY against HT-induced apoptosis and analysed the underlying molecular mechanisms. We incubated human myelomonocytic lymphoma U937 cells at 44 °C for 30 min with or without DMY and followed by further incubation for 6 h at 37 °C. Cell viability was determined by the CCK-8 assay. DMY did not cause any cytotoxic effects in U937 cells even at high doses. HT treatment alone induced significant apoptosis, which was detected by DNA fragmentation and Annexin V/PI double staining. Mitochondrial dysfunction was identified by loss of mitochondrial membrane potential (MMP) during heat stimulation. Apoptotic related proteins were involved, truncated Bid and caspase-3 were upregulated, and Mcl-1 and XIAP were downregulated. We also identified the related signalling pathways, such as the MAPK and PI3K/AKT pathways. However, changes in HT were dramatically reversed when the cells were pretreated with DMY before exposure to HT. Overall, MAPKs and PI3K/AKT signalling, mitochondrial dysfunction, and caspase-mediated pathways were involved in the protective effect of DMY against HT-induced apoptosis in U937 cells, which was totally reversed by DMY pretreatment. These findings indicate a new clinical therapeutic strategy for the protection of immune cells during heatstroke.

Keywords Dihydromyricetin · Hyperthermia · Apoptosis · Heatstroke · Mitochondria

Introduction

Natural compounds are widely used in cosmetics, health care products, and medical treatments. The identification of the effective components and their functions will provide a basis for advances in biochemistry and medicine. Dietary flavonoids are found in many vegetables and fruits, and they have abundant bioactivities that are beneficial to human health. The roles of the pharmacokinetic behaviours of flavonoids in pharmacodynamics and in the use of nutritional supplements with therapeutic effects have been a focus of recent research [1].

Dihydromyricetin (DMY, 3,5,7,3′4′5′-hexahydroxyl-2,3-dihydrogen flavonol, Fig. 1a), is a flavonoid also known as ampelopsin. It is isolated from the leaves and stems of *Ampelopsis grossedentata*. Vine tea, also known as Rattan tea, is prevalent in Southern China and has a high DMY content. DMY possesses a broad range of biological functions; two of the most remarkable are its antioxidant and hepatoprotective effects [2, 3]. In addition, it has biological functions that include hypoglycemic, anti-inflammatory, and neuroprotective effects [4–6]. Based on these benefits, DMY has been widely used as a traditional medicine for many years in China, for alleviating the effects of alcohol, reducing blood pressure and blood lipid levels, and relieving fever. The functions of flavonoids, such as quercetin, myricetin and kaempferol, have been studied extensively. As their

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10495-019-01518-y) contains supplementary material, which is available to authorized users.

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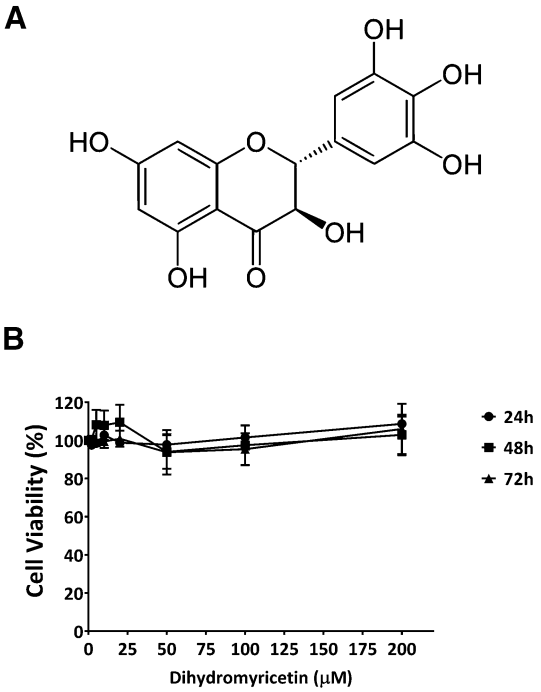


Fig. 1 DMY showed no cytotoxic effects on U937 cells. **a** Chemical structure of dihydromyricetin. **b** U937 cells were treated with the indicated concentrations of DMY for 24, 48, and 72 h, and cell viability was evaluated with the CCK-8 assay. The data are shown as mean values ± S.E.M for independent experiments (n = 3)

structures are very similar to that of DMY, their efficacy and the mechanisms may be comparable. A protective effect of quercetin on heat stroke-induced myocardial injury in male rats has been described by Lin et al. [7]. Based on the finding, we speculated that DMY might have a similar effect, thereby we subsequently explored its potential protective effect against high temperature-induced injury.

The measurement of temperature is a primary method used to determine the condition of the body; the human body is usually maintained at 37 °C. An elevated core temperature is generally considered to be a pathophysiological reaction. Fever is a defensive reaction of an organism and a precursor to certain diseases. Hyperthermia (HT) differs from fever because it is the result of a failure of the body to regulate heat. HT occurs when heat production or absorption exceeds the amount of heat consumed by the body [8]. When HT occurs, microenvironment, nervous system, immune system, and multiple organs, especially the brain, are disrupted. Several common conditions that can lead to HT, including heatstroke, hyperthermia therapy, and malignant hyperthermia (MH) [9, 10]. MH is usually triggered by specific volatile anaesthetic agents or succinylcholine in susceptible individuals [11, 12]. In sensitive individuals, these drugs can cause a severe and uncontrolled increase in oxidative stress in skeletal muscles, accompanied by a more significant ability to supply oxygen, the release of carbon dioxide, the subsequent increase in body temperature (core temperature rises above 44 °C), and immune system dysfunction. If not treated immediately, these conditions may result in circulatory failure and death [13]. People are more prone to heatstroke in hot and humid weather than on regular-temperature days. Heatstroke is a high-risk disease considered a medical emergency [14]. Therefore, daily prevention of heatstroke is critical for people who live in tropical areas where the summer temperatures exceed 40 °C.

In HT, the immune system is challenged by the high temperatures, and cells undergo apoptosis. Immune cells must be protected to maintain lymphocyte function impaired by HT. Lymphocyte apoptosis increases and thereby ameliorates the immune response under the high temperature stress [15]. In the present study, a 44 °C water bath was used to simulate an extreme HT condition, and the U937 cell line was used as a model of lymphocytes. We investigated the protective effect of DMY against HT-induced apoptosis and its associated molecular mechanisms.

Materials and methods

Reagents

Dihydromyricetin (DMY) and SB203580 (a p38 inhibitor) were obtained from Sigma Aldrich (St. Louis, MO). Primary antibodies against Akt (pan), phosphorylated AKT, cleaved caspase-3, LC3A/B, Mcl-1, XIAP, p38, phosphorylated p38, ERK1/2, phosphorylated ERK1/2, HSP27, phosphorylated HSP27 and secondary anti-mouse and anti-rabbit antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-Bcl-2, -JNK, -phospho JNK, -Bax, and -Bid antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-actin antibody, LY294002 (an AKT inhibitor), and U0216 (an ERK inhibitor) were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). JNK inhibitor VIII (a JNK inhibitor) was obtained from EMD Chemicals Inc. (San Diego, CA).

Cell culture

Human myelomonocytic lymphoma U937 cells were procured from the Human Sciences Research Resources Bank (Japan Human Sciences Foundation, Tokyo, Japan). HaCaT cells (human immortalized keratinocytes) were a generous gift from Dr. Ogawa (Department of Radiological Sciences, University of Toyama). U937 cells were maintained in RPMI-1640 (Wako, Osaka, Japan) and HaCaT cells were grown in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum

(FBS) at 37 °C in humidified air with 5% CO_2. The cells were used at log phase for the experiments.

Hyperthermia treatment

Hyperthermia treatment was carried out using a water bath system. Cells (1.5×10^6/sample) were incubated with or without DMY at different concentrations and then immediately transferred to a water bath at 44 °C for 30 min. After HT treatment, cells were directly transferred to an incubator at 37 °C for further incubation.

Measurement of cell viability and cell number

U937 cells (5×10^3) were seeded in 96-well plates and pretreated with DMY at different concentrations (0–200 μM) for 24, 48, and 72 h. For the HT experiment using U937 cells, after exposure to 44 °C for 30 min with or without DMY, cells were seeded in 96-well plates at 5×10^3 cells/well and cell viability was evaluated after 24, 48, or 72 h. HaCaT cells (5×10^3) were seeded in 96-well plates for 24 h and exposed to 44 °C for 30 min with or without DMY. Cell viability was analysed using Cell Counting Kit-8 (CCK-8, Dojindo Lab., Kumamoto, Japan); cells were incubated with 10 μl of solution in an incubator for another 4 h. A Multiskan FC Microplate Photometer (Thermo Scientific, Waltham, MA) was used to measure absorbance at 450 nm. The survival rate was calculated by normalization to the control group. Following primary HT treatment, U937 cells were counted at 24 h intervals up to 96 h, and the medium was refreshed at each time point.

DNA fragmentation

Quantitative DNA fragmentation was assayed as detailed previously [16]. Briefly, the cells were collected and subjected to centrifugation at 2000×*g* for 5 min. After lysis of the pellet in lysis buffer (10 mM Tris, 1 mM EDTA, pH 7.5) containing 0.2% Triton X-100, lysates were centrifuged at 13,000×*g* for 10 min to separate the supernatant and pellet. The supernatant containing fragmented DNA was then transferred to a microcentrifuge tube, and both the pellet and supernatant were precipitated in 12.5% trichloroacetic acid (TCA) overnight at 4 °C. Precipitates were then centrifuged at 13,000×*g* for 10 min after which pellets were heated at 90 °C for 10 min in 5% TCA. Finally, DNA was quantified by measuring absorbance at 600 nm (DU-50; Beckman-Coulter) after diphenylamine (DPA) treatment overnight. The term "percent fragments" refers to the ratio of DNA in the supernatants (obtained by centrifugation at 13,000×*g*) to the total DNA recovered in the supernatants and pellets.

Morphological observation

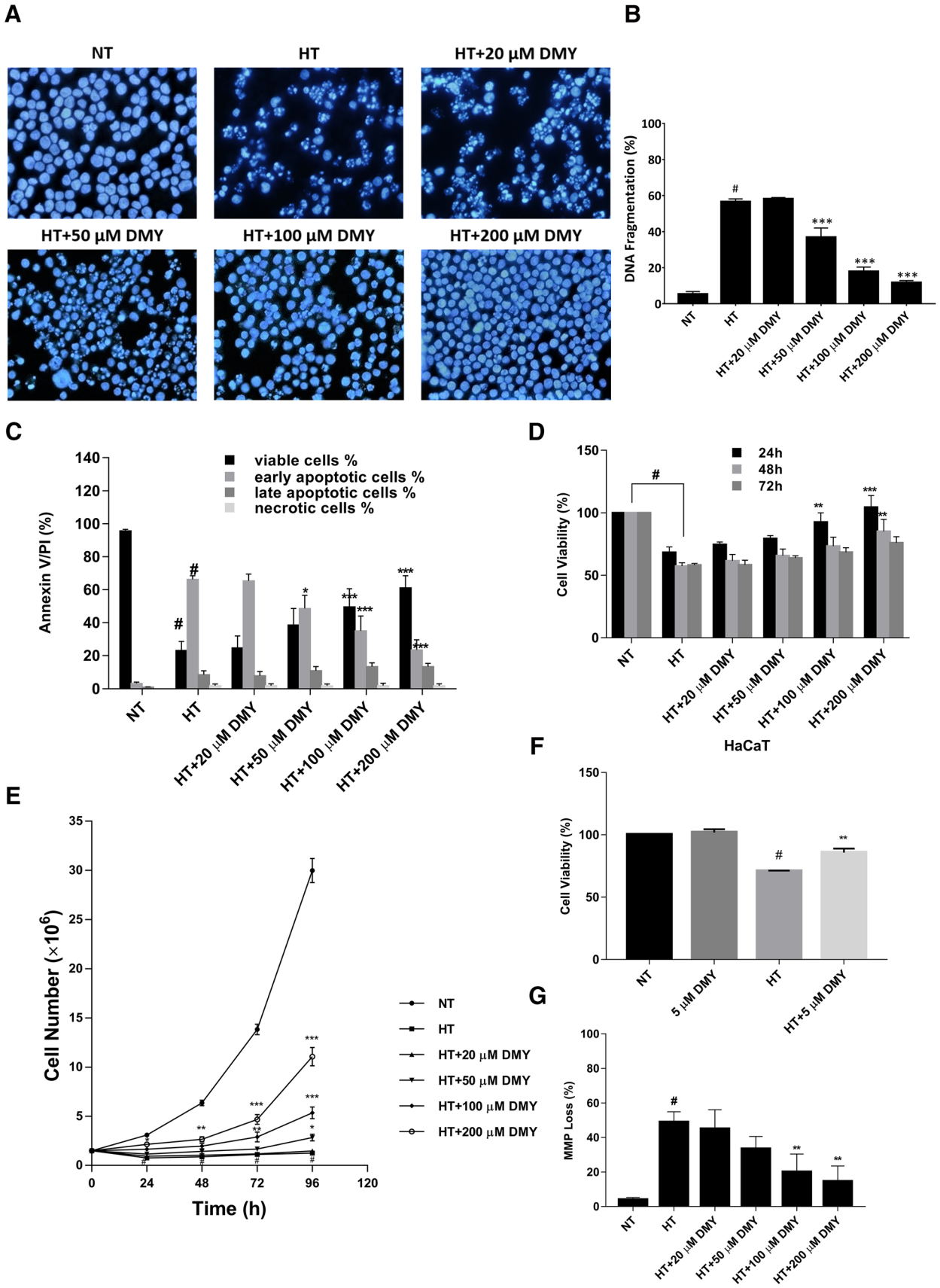
After HT treatment for 6 h, cells were collected and washed with PBS, fixed with 4% paraformaldehyde for 30 min in PBS at 4 °C, and stained with 1 mM Hoechst 33258 for 5 min. Cells were observed under a fluorescence microscope (BX-61; Olympus, Tokyo, Japan) with 20 × objective lens and 10 × eyepiece.

Flow cytometry

To detect apoptosis, after incubation for 6 h, the cells were resuspended in binding buffer (10 mM HEPES, ph 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and stained with Annexin V-FITC and propidium iodide (PI) for 15 min at room temperature in the dark. To detect the loss of mitochondrial membrane potential (MMP), the cells were collected after 4 h of incubation, washed with PBS, and stained with 10 nM tetramethylrhodamine methyl ester perchlorate (TMRM) (Molecular Probes, Eugene, OR) in PBS with 1% FBS for 15 min at 37 °C. After staining, the samples were subjected to flow cytometry (FACSCanto II, BD Biosciences, Mississauga, ON, Canada) according to the manufacturer's instructions [17]. The CellQuest software program (BD Biosciences, San Diego, CA) was used to analyse the data.

Western blot analysis

The treated U937 cells were resuspended in RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) with protease inhibitor cocktail tablets and PhosSTOP (Roche Diagnostics GmbH, Mannheim, Germany) for 20 min on ice. The samples were centrifuged at 13,000×*g* for 10 min, and the supernatants were transferred to new tubes. The protein concentration was measured using the Coomassie (Bradford) Protein Assay Kit (Thermo Fisher Scientific) using samples prepared at equal concentrations with loading buffer. Protein samples were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). Membranes were blocked with 5% skim milk and incubated with a specific primary antibody at 4 °C overnight. After washing with TBST (150 mM NaCl, 50 mM Tris, pH 7.5, 0.1% Tween-20), membranes were incubated with the respective secondary antibodies for 1 h. Protein signals were detected using the ECL solution according to the manufacturer's instructions (Amersham Biosciences, Buckinghamshire, UK).


A
NT
HT
HT+20 μM DMY
HT+50 μM DMY
HT+100 μM DMY
HT+200 μM DMY
B
DNA Fragmentation (%)
C
Annexin V/PI (%)
viable cells %
early apoptotic cells %
late apoptotic cells %
necrotic cells %
D
Cell Viability (%)
24h
48h
72h
HaCaT
E
Cell Number (×10^6)
Time (h)
F
Cell Viability (%)
5 μM DMY
HT+5 μM DMY
G
MMP Loss (%)

◂**Fig. 2** DMY had a protective effect against HT-induced apoptosis via mitochondria dysfunction. U937 cells were treated at 44 °C for 30 min with or without DMY (20 μM, 50 μM, 100 μM, and 200 μM) and further incubation at 6 h at 37 °C. Determined by **a** Hoechst staining, **b** DNA fragmentation, **c** Annexin V/PI staining, **d** CCK-8 assay, and **e** Cell counts. **f** HaCaT cells were heated at 44 °C for 30 min with or without 5 μM DMY and analysed by CCK-8 assay after 24 h. **g** MMP loss was detected by TMRM staining using flow cytometry after 4 h of incubation. #$p < 0.001$ versus NT. **$p < 0.01$ and ***$p < 0.001$ versus HT (n = 3)

Statistical analysis

All results are shown as mean values ± S.E.M for at least three rounds of independent experiments. Differences were compared by one-way analysis of variance (ANOVA) and analysed with Dunnett Hsu's test. $P < 0.05$ was considered significant. Data were analysed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA).

Results

DMY did not have any cytotoxic effects on U937 cells

We treated U937 cells with various concentrations of DMY (0 μM, 20 μM, 50 μM, 100 μM, and 200 μM) for 24, 48, and 72 h and then checked cell viability using the CCK-8 assay (Fig. 1b). The results indicated that DMY had almost no cytotoxic effect on the cells.

DMY had a cytoprotective effect against HT

HT caused apoptosis, nuclear shrinkage, and formed apoptosomes formation prior to cell death. We heated the U937 cells at 44 °C for 30 min with or without DMY. As shown in Fig. 2a, Hoechst staining indicated that HT induced significant nuclear shrinkage and the appearance of apoptosomes. DNA fragmentation assay revealed that a high temperature caused apoptosis in about 56.56 ± 1.57% of cells; pretreatment with DMY protected the cells significantly from heat stress, and apoptosis decreased to 11.66 ± 1.18% in the 200 μM DMY group (Fig. 2b). These results were also confirmed by Annexin V/PI staining. The number of viable cells increased after DMY pretreatment (from 23.16 ± 5.41 to 61.17 ± 7.33%) while the number of apoptotic cells decreased from 66.28 ± 2.17 to 23.47 ± 6.08% (Fig. 2c). We used a DNA ladder to evaluate fragmentation by agarose gel electrophoresis, as shown in the Supplementary Figure; HT group illustrated a clear DNA ladder character, in contrast, pretreated DMY exhibited few apparently ladder pattern or DNA fragmentation. It also proved that DMY can protect DNA damage caused by HT.

The effect of DMY on viability in HT-treated cells was measured at 24, 48, and 72 h using CCK8-assays. Cell viability after HT treatment for 24 h was 68.34 ± 4.30%. DMY protected the cells against HT-induced apoptosis in a dose-dependent manner and significantly increased cell viability from a concentration of 100 μM (92.51 ± 7.42%) (Fig. 2d). Cell proliferation was also evaluated by a cell counting assay. We found that HT treatment caused a sharp and persistent decrease in cell proliferation. DMY did not totally block the effect of HT, but partially restored the proliferative function of cells, especially after 3 days (Fig. 2e).

We also evaluated the effect of DMY on HaCaT cells. After 44 °C 30 min HT and further 24 h of incubation, cell viability was detected; 70.81 ± 0.44% of cells were alive and pretreatment with 5 μM DMY increased cell viability to 85.74 ± 3.21% (Fig. 2f). These data demonstrated that DMY also has a protective role in HT-treated HaCaT cells.

DMY recovered HT-induced MMP loss

Mitochondria play a vital role in the intrinsic apoptotic pathway. Reactive oxygen species (ROS), DNA damage, endoplasmic reticulum (ER) stress, hypoxia, or other stimuli can trigger mitochondrial dysfunction. During apoptosis, pro-apoptotic Bcl-2 family members, including truncated Bid (t-Bid), bind to the mitochondrial outer membrane to directly promote the release of cytochrome *c* [18] and participate in apoptosis. In our study, high-temperature conditions quickly evoked mitochondrial injury and induced MMP loss of 48.80 ± 3.50% (Fig. 2g). Cotreatment with DMY recovered the effect of HT-induced MMP loss (14.41 ± 5.22% in the HT + 200 μM DMY group). Thus, DMY-suppressed HT-induced apoptosis may have a protective effect on mitochondria.

DMY rescued HT-induced changes in the expressions of apoptosis-related proteins

We first examined the expression of Bcl-2 family members and other apoptosis-related proteins. The expression levels of anti-apoptotic proteins Bcl-2 and Bcl-xl and the pro-apoptotic protein Bax did not change in either the HT group or the combination group. However, after treatment with DMY, the HT-induced reductions in Mcl-1 and XIAP, which are also anti-apoptotic proteins, were attenuated in the U937 cells compared with cells treated with HT alone. The expression of t-Bid, also a member of the pro-apoptotic Bcl-2 family, was decreased by DMY pretreatment but was increased in the HT group. These results indicated that DMY suppressed HT via apoptotic related protein expression, especially the Bcl-2 family and XIAP, in U937 cells (Fig. 3).

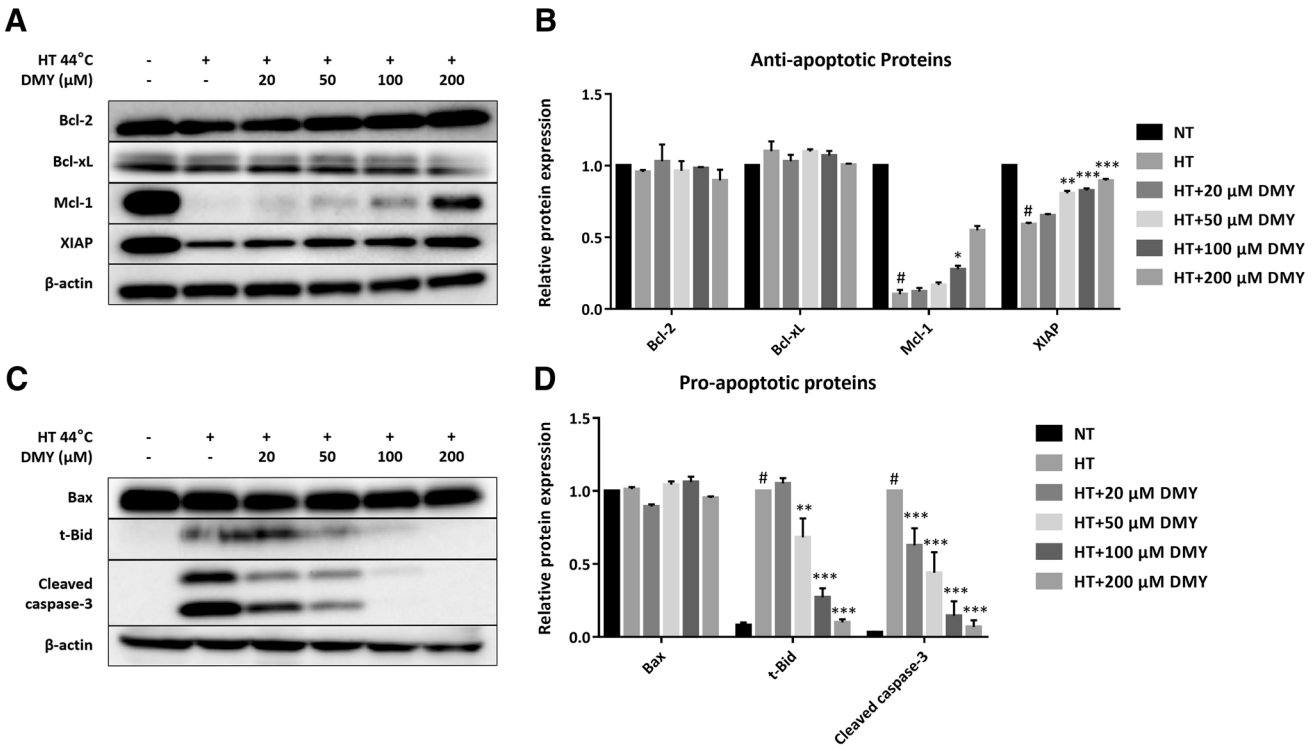


Fig. 3 DMY attenuated HT-induced apoptotic related protein fluctuation. U937 cells were treated at 44 °C for 30 min with or without DMY and further incubated for 6 h at 37 °C. Western blot assay (**a, c**), and quantification of the proteins (**b, d**). Data are shown as mean values ± S.E.M for independent experiments. $^{\#}p < 0.001$ versus NT, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ versus HT; n = 3

DMY protected the U937 cells via the MAPKs and the PI3K/AKT signalling pathways

To identify the upstream signalling pathways, we examined the MAPK and PI3K pathways by western blot analyses. The MAPKs signalling pathway can be activated by HT, and JNK, p38, and ERK are well-known critical targets of the MAPK pathway. The extracellular signalling components translocate to the nucleus, control gene expression, and regulate cell proliferation, cell survival, and apoptosis [19]. HT significantly induced apoptosis in U937 cells by the upregulation of phospho-p38 and phospho-JNK and downregulation of phospho-ERK, while the total amounts of p38, JNK, and ERK remained unchanged. DMY rescued the cells from HT by suppressing changes in the expression of MAPK proteins (Fig. 4a, b). Protein kinase B, also known as AKT, activation is responsible for cell glucose metabolism, cell apoptosis, proliferation, transcription, migration, and invasion [20]. In our study, HT significantly blocked AKT phosphorylation at both threonine 308 and serine 473 in U937 cells. DMY reversed the HT-induced suppression of AKT activation, leading to the promotion of cell survival and proliferation (Fig. 4a, c). These data indicated that DMY-suppressed HT-induced apoptosis might be mediated by the MAPK and PI3K/AKT signalling pathways.

DMY had a similar effect to those of SB203580 and JNK inhibitor VIII in HT-induced apoptosis

To verify the role of p38 and JNK in HT-induced apoptosis, we used SB203580, a p38 inhibitor, and JNK inhibitor VIII. We treated the cells with SB203580 and JNK inhibitor VIII for 1 h before HT and pretreated them with DMY just before exposure to HT. DMY had a similar effect to those of SB203580 and JNK on phosphorylated protein expression, and all of which significantly decreased the expression level of cleaved caspase-3 (Fig. 5a, b, d, e). As shown in Fig. 5c, for SB203580 and DMY, rates of apoptosis were 34.05 ± 1.67% and 19.61 ± 2.14%, respectively, which are remarkably lower than those in the HT group (56.45 ± 3.09%). Furthermore, HT resulted in 50.89 ± 2.27% cell apoptosis, and JNK and DMY resulted in 29.69 ± 0.54% and 22.27 ± 2.61% apoptosis, respectively (Fig. 5f). Therefore, we confirmed that the disruption of p38 and JNK is involved in the effects of DMY against HT-induced apoptosis in U937 cells.

U0126 and LY294002 abrogated the protective effects of DMY

To examine whether ERK and AKT are actually involved in the protective effects of DMY, we pretreated the cells for

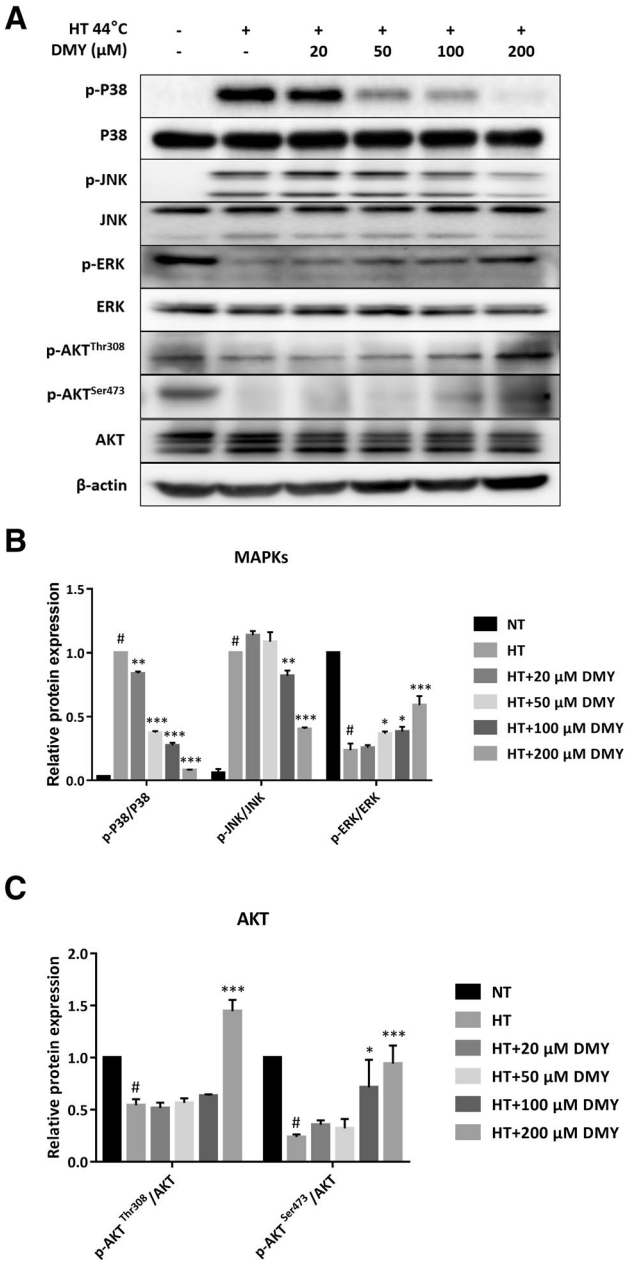


Fig. 4 DMY protected against HT-induced apoptosis through MAPKs and AKT signalling pathways. U937 cells were treated at 44 °C for 30 min with or without DMY and further incubated for 1 h at 37 °C. Western blot assay (**a**), and quantification of the proteins (**b, c**). Data are shown as mean values ± S.E.M for independent experiments. $^{\#}p < 0.001$ versus NT, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ versus HT; n = 3

1 h with or without U0126 and LY294002, which are inhibitors of ERK and AKT, respectively. After pretreatment with each inhibitor, DMY was added to the cells and cells were exposed to HT. Phosphorylated ERK and AKT were both markedly decreased in the HT group, while cleaved caspase-3 was activated. Nonetheless, DMY attenuated caspase-3 activation, and HT-induced ERK and AKT suppression. In the presence of inhibitors, the expressions of p-ERK and p-AKT were blocked entirely with consequent activation of cleaved caspase-3, and a significant increase in DNA fragmentation was observed compared with that in the DMY group (from 14.21 ± 1.76 to 20.83 ± 0.43% and 21.84 ± 1.49 to 32.42 ± 2.93%, respectively) (Fig. 6). These results proved that DMY protects cells against HT-induced apoptosis by restoring the activation of ERK and AKT.

DMY relieved the heat stress as evidenced by the expression of HSP27 phosphorylation

The phosphorylation of HSP27 is downstream of p38; thus, we tested the expression levels of p-HSP27 and total HSP27. HT induced stress and triggered HSP27 phosphorylation, while DMY prevented p-HSP27 accumulation in a dose-dependent manner; total HSP27 was not involved in this process (Fig. 7a, b). Similarly, pretreatment with SB203580 also blocked p-HSP27 (Fig. 7c, d). Thus, p-p38 activated HSP27 in response to heat stress and resulted in the phosphorylation of HSP27.

Discussion

As reported in the *Compendium of Materia Medica*, vine tea extract helps to alleviate the effects of heat, soothes a sore throat, reduces diuresis, and promotes the circulation of blood. It is also functionally involved in the treatment of high temperature responses [21]. The mechanism underlying the effects of DMY on the response to HT is not well understood. According to many reports, DMY has no cytotoxic effects on healthy cells [22, 23]. Based on these functions and benefits, we chose DMY for the prevention of HT in our experiments. To explore the effects of DMY, we used U937 cells as model immune cells. Our results revealed that HT, via the MAPK (p38, JNK, and ERK) and PI3K (AKT) signalling pathways, results in the downregulation of anti-apoptotic Mcl-1, and XIAP and the upregulation of pro-apoptotic t-Bid leads to MMP loss and activation of cleaved caspase-3, which subsequently induces apoptosis. HT also activate HSP27 due to heat stress. DMY reversed these effects and protected the cells against HT-induced apoptosis (Fig. 8).

In our study, we demonstrated that DMY has a protective effect against HT damage. We found no cytotoxicity at doses of up to 200 μM DMY in U937 cells. HT markedly induced DNA fragmentation and subsequent apoptosis. Additionally, cell proliferation was reduced by HT treatment, and the injury lasted several days. However, DMY blocks cell death, weakens the damage, and restores the proliferative function of the cell. Furthermore, we detected a protective effect in HaCaT cells, human immortalized keratinocytes,

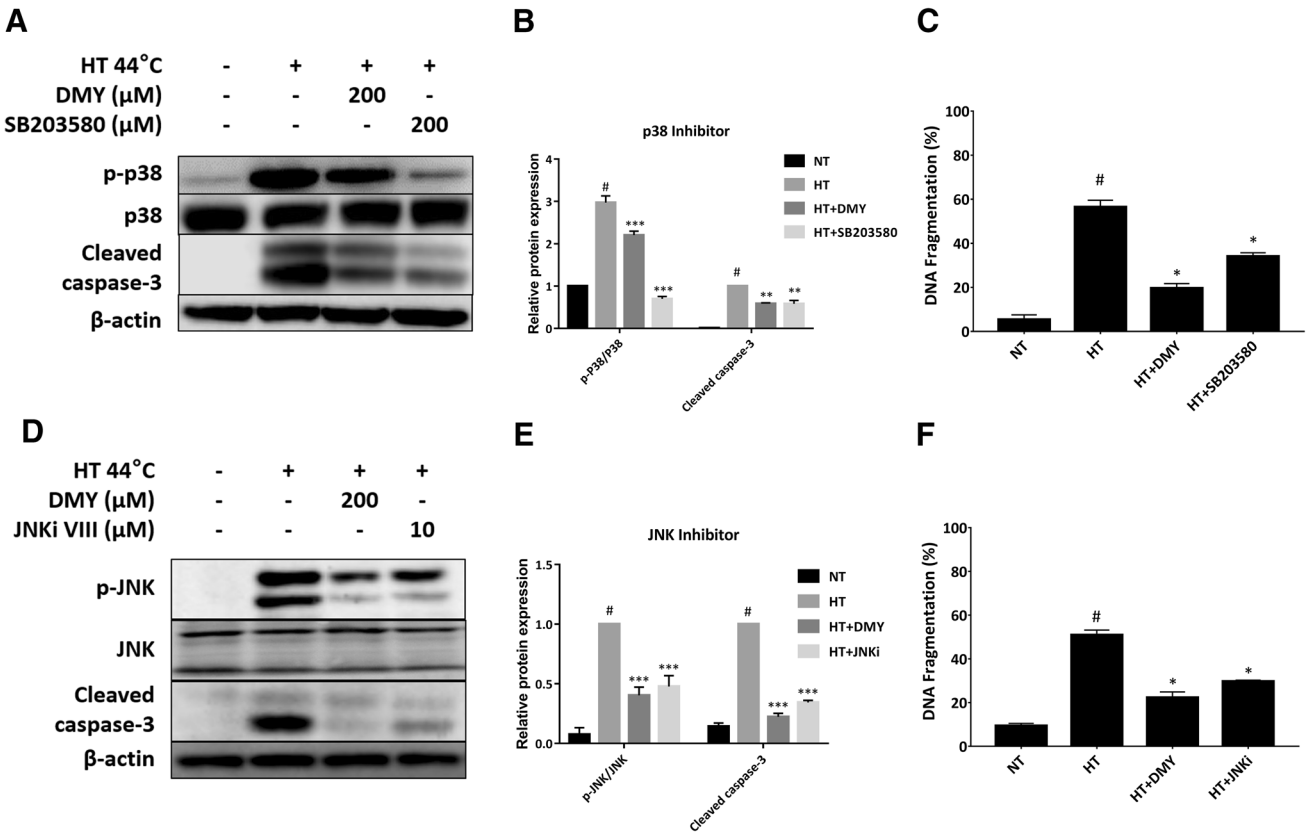


Fig. 5 DMY had similar effects as SB203580 and JNK inhibitor VIII against HT-induced apoptosis. U937 cells were pretreated with 200 μM SB203580 (**a**–**c**, p38 inhibitor), and 10 μM JNK inhibitor VIII (**d**–**f**, JNK inhibitor) for 1 h or DMY just before HT. Expression levels of p-p38, p-JNK, and cleaved caspase-3 were measured using western blot with β-actin as a loading control, with quantification of phosphorylated proteins and cleaved caspase-3 (**a, b, d, e**). Apoptosis was detected by DNA fragmentation (**c, f**). Data are shown as mean values ± S.E.M for independent experiments. $^{\#}p < 0.001$ versus NT, $^{**}p < 0.01$ and $^{***}p < 0.001$ versus HT; n = 3

in which DMY had a similar effect against HT-induced cell death. Mitochondria are the engine of the cell; mitochondrial dysfunction is considered to be an early event during most cell alterations [24–26]. To detect the disruption of normal mitochondrial function, we evaluated the loss of MMP. Our obtained data showed that HT treatment of the cells caused a loss of MMP. However, MMP was retained when DMY was applied before HT treatment. Hence, we speculate that the protective effect of DMY on mitochondria is the most critical pathway in preventing HT-induced apoptosis.

Myeloid cell leukaemia 1 (Mcl-1) is an anti-apoptotic member of the Bcl-2 family. It is related to the pro-apoptotic member BH3, an interacting domain death agonist (Bid). Cleavage of caspase-3 renders Mcl-1 proapoptotic. Moreover, in cooperation with tBid, it also promotes mitochondrial apoptosis [27]. Another anti-apoptotic protein, X-linked inhibitor of apoptosis protein (XIAP), inhibits apoptosis by binding to cell death enzymes caspase-3, 7, and 9 [28]. After pretreatment with DMY, HT-suppressed XIAP was recovered. Furthermore, DMY also blocked HT-induced activation of caspase-3, and hence, cell death decreased. The Bcl-2 family generally plays a key role in cell apoptosis. However, Bcl-2, Bcl-xL, and Bax levels showed almost no change in either the HT group or the combination group. HT at 44 °C for 30 min has been evaluated in the past, and cell lysate Bcl-2, Bcl-xL, and Bax are unchanged while mitochondrial Bax is overexpressed through HT treatment [29]. One possible explanation is that Bax translocates to the mitochondria, promoting cytochrome *c* release and then triggeres caspase cascades [30]. At the same time, it did not affect the Bax level in whole cell lysates. In a further study, we will confirm this speculation.

MAPK is an essential transmitter of signal transduction from the cell surface to the nucleus involved in gene expression, regulation, and cytoplasmic function. The MAPK pathway is also the primary controller of key processes, such as cell differentiation, cell proliferation, and cell death [19]. As indicated in a previous report, p38 and JNK are two kinds of stress-activated protein kinases [31]. It was found that HT induced the phosphorylations of p38 and JNK. ERK has opposite effects to those of p38 and JNK on apoptosis, as MKK1/2 activates ERK to promote cell survival [31].

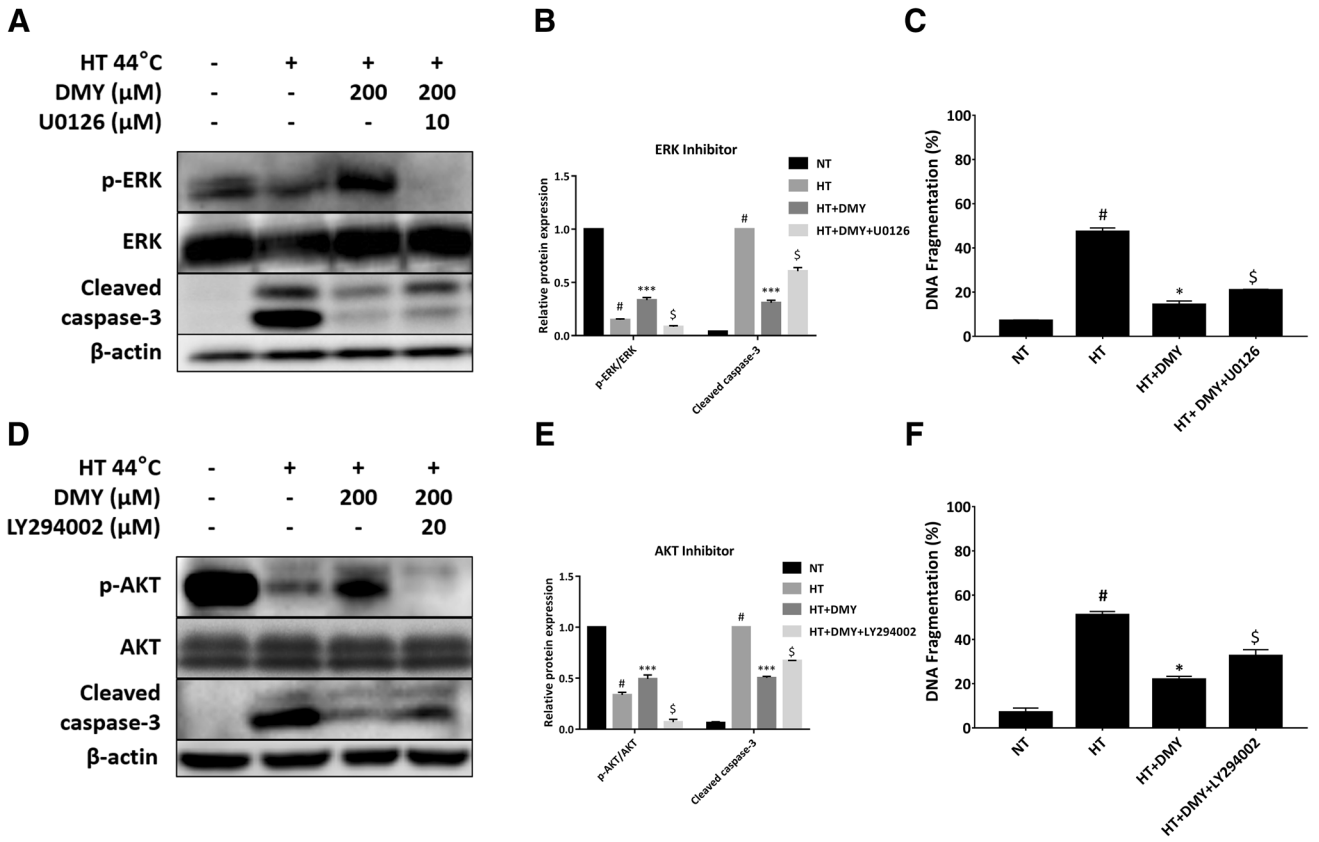


Fig. 6 DMY inhibition of HT-induced apoptosis was abrogated by U0126 and LY294002. U937 cells were pretreated with 10 μM U0126 (**a**–**c**, ERK inhibitor) or 20 μM LY294002 (**d**–**f**, AKT inhibitor) for 1 h and then treated at 44 °C for 30 min with or without DMY. Expression levels of MAPK and AKT were measured by western blotting with β-actin as a loading control; phosphorylated proteins and cleaved caspase-3 were quantified (**a, b, d, e**). Apoptosis was detected with DNA fragmentation (**c, f**). Data are shown as mean values ± S.E.M for independent experiments. $^{\#}p < 0.001$ versus NT, $^{***}p < 0.001$ versus HT, $^{\$}p < 0.05$ and ns. versus HT + DMY; n = 3

The PI3K/AKT signalling pathway regulates cell cycle and cell survival. The phosphorylation of multiple substrates of AKT has been shown to block apoptosis via the regulation of transcriptional activity [32]. Phospho-ERK and phospho-AKT (serine 473 and threonine 308) were suppressed by HT and inhibited cell viability. DMY reversed these effects. These data indicated that MAPK and PI3K/AKT signalling pathways were both involved in DMY-mediated protection against HT-induced apoptosis.

Heat shock proteins (HSPs) are stress-inducible proteins that respond to high temperatures and other stimuli. These include conditions such as hypoxia and ischaemia. HSPs are named by their molecular weight, such as HSP27, HSP70, and HSP90 [33]. Heat shock protein 27 (HSP27), also called HSPB1, is a small HSP. It is directly or indirectly involved in the regulation of apoptosis and participates in the management of the cytoskeleton [34]. HSP27 can be activated by p38 under stress [35], causing a rapid increase and accumulation of the phosphorylated form. The use of SB203580, a p38 inhibitor, blocks p38-induced MAPKAPK2 and HSP27 phosphorylation [36]. Interestingly, the phosphorylation of HSP27 can suppress cell growth in some cancer cell lines, such as pancreatic cancer [37] and hepatocellular carcinoma [38, 39]. Consistent with the findings from previous studies, HSP27 was phosphorylated through HT treatment. However, p-HSP27 was inhibited and significantly reduced to the basic level by pretreatment with DMY or SB203580. Collectively, the reduction of HSP27 phosphorylation may be associated with the protection of DMY against heat stress.

In summary, our study demonstrated that DMY protected against HT-induced apoptosis via a mitochondria-caspase-dependent pathway in U937 cells. The protective effect was mediated by the MAPK and PI3K/AKT signalling pathways, which reversed MMP loss and apoptotic related proteins and thus rescued the cells from high temperatures. In future studies, healthy immune cells will be used and in vivo experiments will be carried out after exposure to HT. Our studies offer a new chemopreventive strategy for treating HT and could provide an option for the daily prevention of heatstroke.

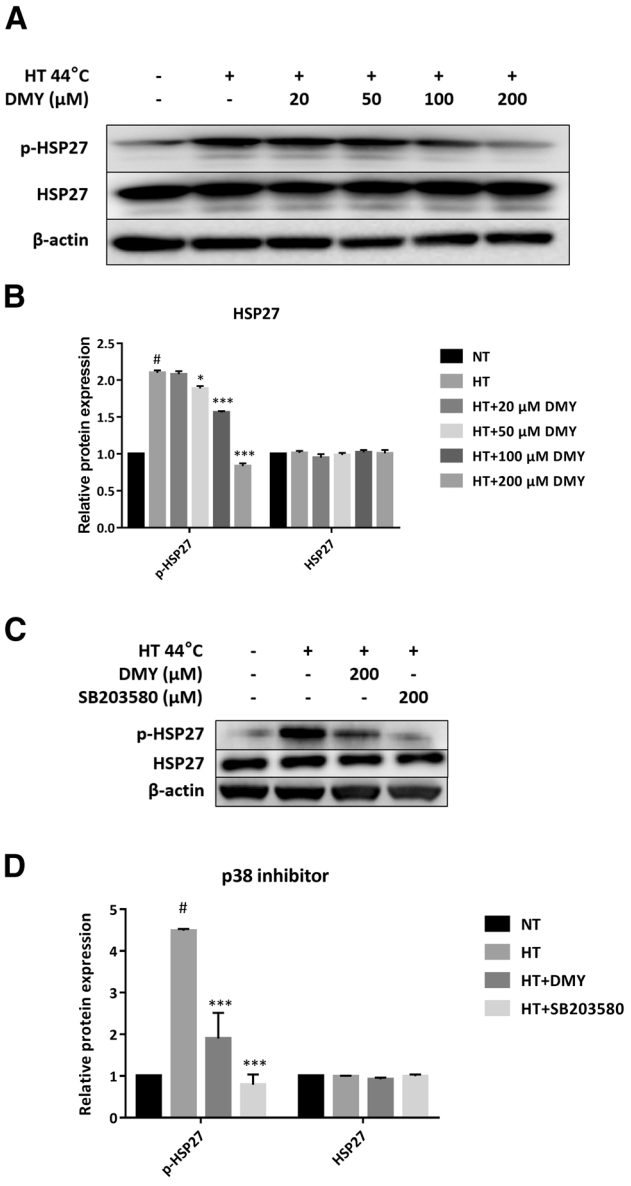


Fig. 7 DMY relieved the stress caused by HT in the expression of HSP27. U937 cells were treated at 44 °C for 30 min with or without DMY and further incubated for 1 h at 37 °C. Western blot assays were performed (**a, c**), and quantification of the proteins (**b, d**). The data are presented as mean values ± S.E.M for independent experiments. $^{\#}p < 0.001$ versus NT, $^{*}p < 0.01$ and $^{***}p < 0.001$ versus HT; n = 3

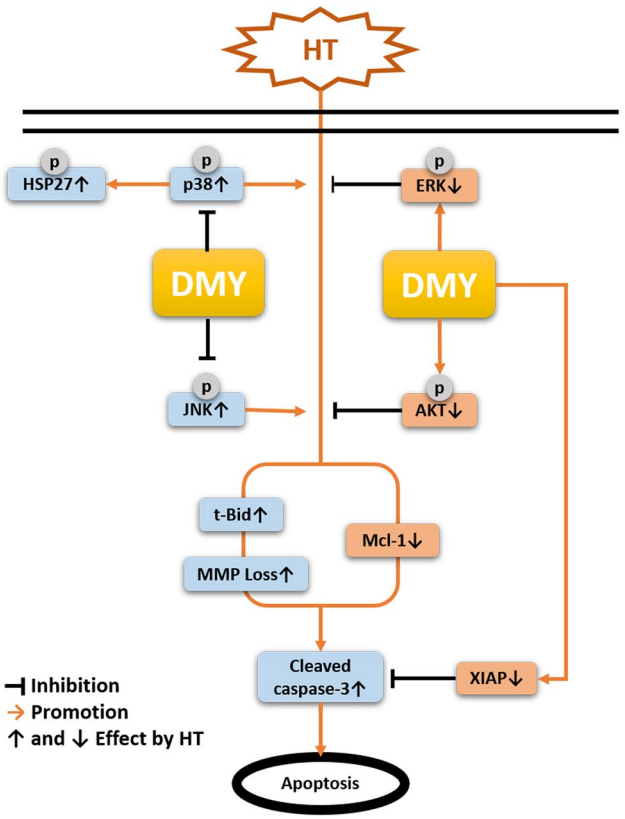


Fig. 8 The proposed mechanism by which DMY inhibited HT-induced apoptosis. Briefly, HT stimulated the MAPK (p38, JNK, and ERK) and PI3K (AKT) signalling pathways. HT caused a decrease in the anti-apoptotic proteins Mcl-1 and XIAP, and an increase in pro-apoptotic protein t-Bid and anti-proliferation protein p-HSP27. Finally, mitochondrial dysfunction and cleavage of caspase-3 led to apoptosis. DMY prevented HT-induced apoptosis by reversing these effects

Acknowledgements This work was supported by JSPS KAKENHI Grant No. 18K10044.

Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interests regarding the publication of this article.

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