Pharmacological Reports 68 (2016) 275–282

Contents lists available at ScienceDirect

Pharmacological Reports

journal homepage: www.elsevier.com/locate/pharep

Original research article

The heat shock protein 90 inhibitor, 17-AAG, attenuates thioacetamide induced liver fibrosis in mice

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ARTICLE INFO

Article history:
Received 9 April 2015
Received in revised form 28 August 2015
Accepted 31 August 2015
Available online 11 September 2015

Keywords:
Heat shock protein
17-AAG
Apoptosis
Liver fibrosis

ABSTRACT

Background: Heat shock protein 90 (Hsp90) is proposed to be involved in liver disorders. This study was conducted to test effect of 17-N-allylamino-17-demethoxygeldanamycin (17-AAG), an inhibitor of Hsp90, on attenuating thioacetamide induced liver fibrosis *in vivo*.
Methods: Four groups of Swiss albino male mice (CD-1 strain) were used as follows: control group; thioacetamide group (received 100 mg/kg thioacetamide, *ip* injection, 3 times/week for 8 weeks); thioacetamide plus 17-AAG groups (received 100 mg/kg thioacetamide, *ip* injection, 3 times/week for 8 weeks plus 25 or 50 mg/kg 17-AAG, *ip* injection, 5 days/week along the last 4 weeks). Fibrosis was quantified by measuring hydroxyproline level and by morphometry and oxidative stress biomarkers were assigned. Relative hepatic mRNA expressions of α-smooth muscle actin (α-SMA), collagen-1-alpha-1 (Col1A1) and tissue inhibitor metalloproteinase-1 (TIMP-1) mRNAs were measured by RT-PCR. Levels of the apoptotic markers caspase-3, factor related apoptosis (Fas) and Hsp-90 were assigned in tissue homogenate.
Results: 17-AAG (50 mg/kg) significantly decreased fibrosis percentage significantly ($p < 0.001$, 0.05) compared to thioaceatmide and 25 mg/kg, respectively. Malondialdehyde, Hsp90, α-SMA, Col1A1 and TIMP-1 expression levels were significantly reduced ($p < 0.05$) by the inhibitor large dose. Levels of GSH, caspase-3 and Fas were markedly ($p < 0.001$) increased in the group received 17-AAG (50 mg/kg) compared to other groups.
Conclusion: The Hsp90 inhibitor, 17-AAG, can attenuate thioacetamide hepatotoxicity through oxidative stress counterbalance, reducing stellate cells activity and inducing apoptosis.

Introduction

Synthesis of distinct number of stress proteins known as heat shock proteins (Hsp) is increased in response to sudden changes in the environmental temperature by certain cells. In addition to their function under stress conditions, they also have a physiological role. According to their molecular weight, Hsp are classified into Hsp10, Hsp40, Hsp70, Hsp90 and others. They act as chaperones with definite roles in protein folding, assembly and transport associating cell-cycle control and protection against stress [1,2].

One of these proteins is Hsp90 that was originally discovered with more abundance upon heat stress. Away from cellular stress, it is also extensively found accounting for about 1–2% of the total soluble cytosolic protein in unstressed eukaryotic cells [3]. It activates and complexes with a variety of normal cellular proteins as glucocorticoid receptors, cytoskeletal proteins, factors inducing hypoxia and tyrosine kinases [4,5].

Various clinical trials were conducted to test the anti-tumor effect of Hsp90 inhibitors either alone or in combination with well-known chemotherapeutic agents. They have the ability to control multiple signaling pathways that promote cancer cell survival and maintain malignant transformation. It was found that cancer cells overexpress Hsp90 that in turn interacts with tyrosine kinases

Abbreviations: 17-AAG, 17-N-Allylamino-17-demethoxygeldanamycin; Col1A1, collagen-1-alpha1; GSH, reduced glutathione; HSC, hepatic stellate cells; Hsp, heat shock protein; SMA, smooth muscle actin; TIMP, tissue inhibitor metalloproteinase.

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http://dx.doi.org/10.1016/j.pharep.2015.08.015
1734-1140/

induced by oncogens [6]. Clinical use of initial Hsp90 inhibitors as the naturally occurring products geldanamycin and radiciol was limited because of their poor *in vivo* activity and toxicity [7,8]. As a result, analogs for geldanamycin as 17-N-allylamino-17-demethoxygeldanamycin (17-AAG) and 17-Dimethylaminoethylamino-17-demethoxygeldanamycin were introduced with better binding affinities and less toxicity [9]. In addition to cancer research, many other inhibitors were tested in different diseases and disorders. For example, blocking Hsp90 by EC144 was applied in treatment of autoimmune diseases involving inflammation [10]. In atherosclerosis, cardiovascular diseases and lung injury inhibitors of Hsp90 were capable of attenuating inflammatory responses [11–13].

Concerning liver, previous studies proposed involvement of Hsp90 inhibition in managing some liver disorders. Some inhibitors demonstrate competence in hepatocellular carcinoma approaches [14,15]. Other inhibitors were suggested to alleviate lipopolysaccharide-mediated liver injury through repressive action of heat shock transcription factor 1 and the reduction of pro-inflammatory cytokines [16]. Likewise, Hsp90 was suggested to be involved in stabilization of replication proteins of hepatitis virus C and B [17,18]. In a previous *in vitro* study demonstrated by [19], Hsp90 was found to be involved in activation and survival of stellate cells. The present study novelty is to investigates the *in vivo* effect of the Hsp90 inhibitor 17-AAG on thioacetamide induced liver fibrosis model and its possible anti-fibrotic mechanisms. To mimic the clinical application of anti-fibrotic drugs, 17-AAG doses were administered at an apparent stage of fibrosis (4 weeks after thioacetamide intoxication).

Materials and methods

Animals

Adult male Swiss albino mice (CD-1 strain, $n = 55$, 30–35 gm) were purchased from the Holding Company for Biological Products and Vaccines, VACSERA (Agouza, Giza, Egypt). Mice were allowed free water and food access. The care and use of experimental animals during the study comply with the ethical principles and guidelines adopted by the Scientific Research Ethics Committee of Faculty of Pharmacy, Mansoura University.

Drugs and chemicals

Thioacetamide, 4-hydroxy-L-proline and 5,5′-dithiobis-(2-nitrobenzoic acid) were purchased from Sigma–Aldrich (St. Louis, MO, USA). 17-AAG was purchased as powder from LC labs (New Boston, USA). Reduced glutathione (GSH) was purchased from FlukaChemie (Buchs SG, Switzerland).

Experimental design

Liver fibrosis was induced by injecting thioacetamide (100 mg/kg, 12% w/v in saline, *ip*, 3 times/week) for 8 weeks [20]. The tested inhibitor, 17-AAG, was suspended in a purified phospholipid vehicle prepared as describe by [21]. Four groups were used: control group (received drug vehicle, *ip*, 10 mL/kg/day); thioacetamide treated group ($n = 15$), 17-AAG treated groups ($n = 15$ each, 25 and 50 mg/kg, *ip*, 0.04% w/v, 10 and 20 mL/kg/day) concurrently with thioacetamide during the last 4 weeks. Tested doses were identified as sub-toxic doses in a preliminary trial.

By the end of week 8, mice were anesthetized with thiopental (70 mg/kg, *ip*). Liver was isolated and cut into sections. One section was fixed in 10% (v/v) neutral buffered formalin solution for 24 h for histopathological evaluation. Other sections were used for the preparation of homogenates (10% w/v in phosphate buffer, pH 7.5).

Histopathological examination and fibrosis quantification

Standard histopathological techniques were followed for processing the fixed liver tissue and preparation of paraffin blocks. Quantitative analysis of collagen fiber deposition in *Masson trichrome* stained liver sections was performed by morphometric analysis [22]. Briefly, a total of 10 fields were randomly chosen per mouse and images were taken with a digital camera mounted on a BX51 Olympus optical microscope (Olympus Corporation, Tokyo, Japan). Collagenous areas were extracted from the whole tissue areas and analyzed using the NIH Image software (Scion Corp., Frederick, MD, USA). The extent of peri-vascular fibrosis was expressed as the percentage of the stained area relative to the total area.

Liver content of 4-hydroxyproline was quantified as described by [23] with some modifications. Briefly, liver specimens (50 mg) were hydrolysed in 0.5 mL 6 mol/L NaOH for 2 h at 100 °C. The hydrolysate was then cooled and centrifuged at $8000 \times g$ for 10 min. Then, 200 μl supernatant was added to 0.125 mL chloramine T solution (1 part of 7% chloramine T + 4 parts citrate/acetate buffer pH 6.0) and incubated at room temperature for 10 min. After incubation, 0.75 mL Ehrlich's solution (2 g P-dimethyl amino-benzaldehyde in 3 mL of 60% $HClO_4$ mixed with 9 mL of isopropanol) was added. The reaction mixture was incubated at 60 °C for 35 min and then at room temperature for 10 min. Absorbance was measured spectrophotometrically at 560 nm. Standard curve was constructed for 4-hydroxy-L-proline solution (0, 20, 40, 60, 80 and 100 μg/mL) and used to determine hepatic 4-hydroxyproline content, expressed as μg/g tissue.

Immunohistochemistry of caspase-3

Paraffin-embedded sections were deparaffinized with xylene and dehydrated in decreasing concentrations of ethanol. To block endogenous peroxidase activity, sections were treated with 3% hydrogen peroxide in methanol for 10 min. Tissue sections were processed in EDTA for antigen retrieval and incubated for 1 h at 37 °C with caspase-3 monoclonal antibodies (H-60, Santa Cruz, CA, USA). The best dilution ratio (1/100) was determined in multiple experiments and all procedures were performed at room temperature according to the manufacturer's instructions using HRP-DAB detection system. Binding was visualized with 3,3′-diaminobenzidine and slides were examined after counterstaining with hematoxylin. Sections were examined by light microscope using a high objective for caspase-3 positive cells at the deposition sites of collagen in the portal and periportal areas as well as parenchymal sinusoidal location.

Evaluation of oxidative stress and antioxidant biomarkers

Lipid peroxidation was indirectly evaluated by measuring hepatic tissue malondialdehyde level as previously demonstrated by [24]. Hepatic GSH level was determined as described by [25] with some modification. In brief, 0.05 mL of 50% (w/v) trichloroacetic acid was added to 0.45 mL of liver homogenate to precipitate protein then centrifuged at $1000 \times g$ for 5 min. 0.25 mL of supernatant was mixed with 1 mL of 0.2 M Tris–HCl (containing 1 mM EDTA, pH 8.9) and 0.05 mL of 0.01 M 5,5′-dithiobis-(2-nitrobenzoic acid) in absolute methanol and kept at room temperature for 5 min. The yellow color developed was measured spectrophotometrically at 412 nm. A standard curve (0–500 nmol/mL) was constructed and results were determined and expressed as nmol/g tissue.

RNA extraction, cDNA synthesis, and RT-PCR

Liver tissue expression of α-smooth muscle actin (α-SMA), collagen-1-alpha-1 (Col1A1) and tissue inhibitor metalloproteinase-1 (TIMP-1) mRNAs was evaluated by RT-PCR. Total RNA was extracted from liver tissue grounded to powder in liquid nitrogen using column technique according to the manufacturer's instructions (Vivantis Technologies Sdn. Bhd., Kuala Lumpur, Malaysia). Then, 1 μg of total RNA was reverse transcribed to cDNA by Maxima first strand cDNA synthesis kit (Thermo Scientific, Canada). 2 μl of the cDNA was amplified in the thermal cycler instrument (Arktik Thermal Cycler, CA, USA) after adding 20 pmol of each primer pair, 10 μl of 2× Taq PCR Master Mix (TaKaRa Bio Inc., Japan), and nuclease-free water to reach a total volume of 20 μl. The primers sequences designed: For Col1A1: 5′-CAGGCTGGTGTGATGGGATT-3′ (sense) and 3′-GACCACGGGCAC-CATCTTTA-5′ (antisense); for α-SMA: 5′-GGCATCCACGAAAC-CACCTA-3′ (sense) and 3′-TGGAAGGTAGACAGCGAAGC-5′ (antisense); for TIMP-1: 5′-AGAGACACACCAGAGCAGAT-3′ (sense) and 3′-ACCGGATATCTGCGGCATTT-5′ (antisense). β-actin was included as a reference gene with the sequence: 5′-CAGGATTCCA-TACCCAAGAAG-3′ (sense) and 3′-AACCCTAAGGCCAACCGTG-5′ (antisense). PCR amplification cycles: initial denaturation at 95 °C for 5 min; then 35 cycles for Col1A1 and TIMP-1, 30 cycles for β-actin at 94 °C for 30 s (denaturation), at 60 °C for 30 s (annealing) and at 72 °C for 45 s (extension). The final extension was carried out at 72 °C for 5 min. 10 μl of PCR products was separated by electrophoresis on 2% TAE agarose gel (Vivantis Technologies Sdn. Bhd., Kuala Lumpur, Malaysia) visualized by ethidium bromide stain (Sigma, MO, USA) and 100-bp DNA ladder (Vivantis Technologies Sdn. Bhd., Kuala Lumpur, Malaysia) was applied in the first lane to identify the molecular weight. Gel was photographed and analyzed by Gel Documentation system (Bio-Rad, CA, USA).

ELISA's

Hepatic tissue levels of caspase-3, factor related apoptosis (Fas) (USCN Life Science Inc., Wuhan, China) and Hsp90 (CUSABIO, Wuhan, China) were measured according to the manufacturers' instructions.

Statistical analysis

Data are expressed as mean ± SE in each experimental group ($n = 8$). Statistical evaluations of the results were carried out by means of one way analysis of variance, followed by the Tukey–Kramer multiple comparison test. Statistical tests and graphs were performed using GraphPad Prism V5.01 (GraphPad Software Inc, San Diego, CA, USA).

Results

Administration of thioacetamide alone resulted in high percentage mortality (40%) by the end of the study. Concurrent administration of 17-AAG (25 or 50 mg/kg) decreased mortality percentage to ~27 and 13% respectively. Isolated liver from thioacetamide group was noticed to have rough surface with small nodules compared to control group. Staining of liver sections from control mice with *Masson Trichrome* revealed typical collagen distribution pattern (in the central and portal vessels). However, liver sections isolated from mice received thioacetamide alone showed a significant periportal fibrosis with portal–portal septa framing liver lobules. Less apparent collagen deposition was observed in 17-AAG (50 mg/kg) group. By examination of hematoxylin–eosin stained sections, hepatocytes degeneration and inflammatory cells infiltration were found to be minimized by the Hsp90 inhibitor (50 mg/kg). Examination of immuno-stained sections revealed caspase-3 positive cells more apparently in portal and periportal areas where collagen deposition is observed (active HSCs) rather than in parenchymal cells (hepatocytes) (Fig. 1A).

Effect on fibrosis area and hydroxyproline content

The grading method applied showed a significant ($p < 0.001$) high %fibrosis in thioacetamide group compared to control group. The percentage was significantly decreased by 17-AAG (25 and 50 mg/kg, $p < 0.001$) where the higher dose was more efficient in conserving liver architecture (Fig. 1B). Hydroxyproline level was significantly ($p < 0.001$) lowered by 17-AAG (50 mg/kg) compared to thioacetamide and 25 mg/kg received groups (Fig. 1C).

Effect on hepatic stellate cells (HSCs) activity and Hsp90 hepatic level

Hepatic COL1A1 mRNA expression was assessed by RT-PCR where COL1A1/β-actin band intensity ratio was determined and expressed as relative density. Thioacetamide administration resulted in about two fold increase in COL1A1 expression (Fig. 2A and D). This elevated level was decreased by 17-AAG (50 mg/kg) more significantly ($p < 0.05$) than by the small dose (25 mg/kg).

Expression of hepatic α-SMA was assigned to estimate activity of hepatic fibroblasts. Thioacetamide increased mRNA expression by 1.6 fold compared to control group. The α-SMA expression level was found to be significantly ($p < 0.001$, 0.05) low in 17-AAG (50 mg/kg) received group when compared to groups received thioacetamide and 17-AAG (25 mg/kg), respectively (Fig. 2B and E). Hepatic expression of TIMP-1 as well was significantly changed in thioacetamide received group (12.4 fold increase, $p < 0.001$). Injection of 17-AAG (25 or 50 mg/kg) decreased TIMP-1 expression by 1.5 and 2.4 folds respectively compared to expressed level in thioacetamide received group (Fig. 2C and F).

The apoptotic marker caspase-3 was significantly ($p < 0.001$) increased in 17-AAG (50 mg/kg) received group when compared to thioacetamide and small dose (25 mg/kg) received groups (Fig. 3A). Similar results for Fas level were obtained as the tested Hsp90 inhibitor (50 mg/kg) significantly ($p < 0.001$) decreased Fas level when compared to levels in thioacetamide and 17-AAG (25 mg/kg) received groups (Fig. 3B).

Injection of thioacetamide for 8 weeks increased Hsp90 tissue level significantly ($p < 0.001$) compared to control group. Injection of 17-AAG (25 or 50 mg/kg) concurrently with thioacetamide decreased the measured protein level ($p < 0.001$) significantly. In group received the small dose (25 mg/kg), Hsp90 level remained significantly ($p < 0.001$) higher than in control and 17-AAG (50 mg/kg) received groups (Fig. 3C).

Oxidative stress biomarkers

Tissue level of malondialdehyde was significantly ($p < 0.001$) increased by thioacetamide administration. Both tested doses of 17-AAG significantly ($p < 0.001$) lowered malondialdehyde tissue level compared to thioacetamide group level (Fig. 4A). On the other hand, GSH level was elevated significantly ($p < 0.01$, 0.001) by 17-AAG (25 and 50 mg/kg respectively) compared to thioacetamide received group (Fig. 4B).

Discussion

Thioacetamide is a hepatotoxin that has been experimentally used to study liver fibrosis and its underlying mechanisms as it

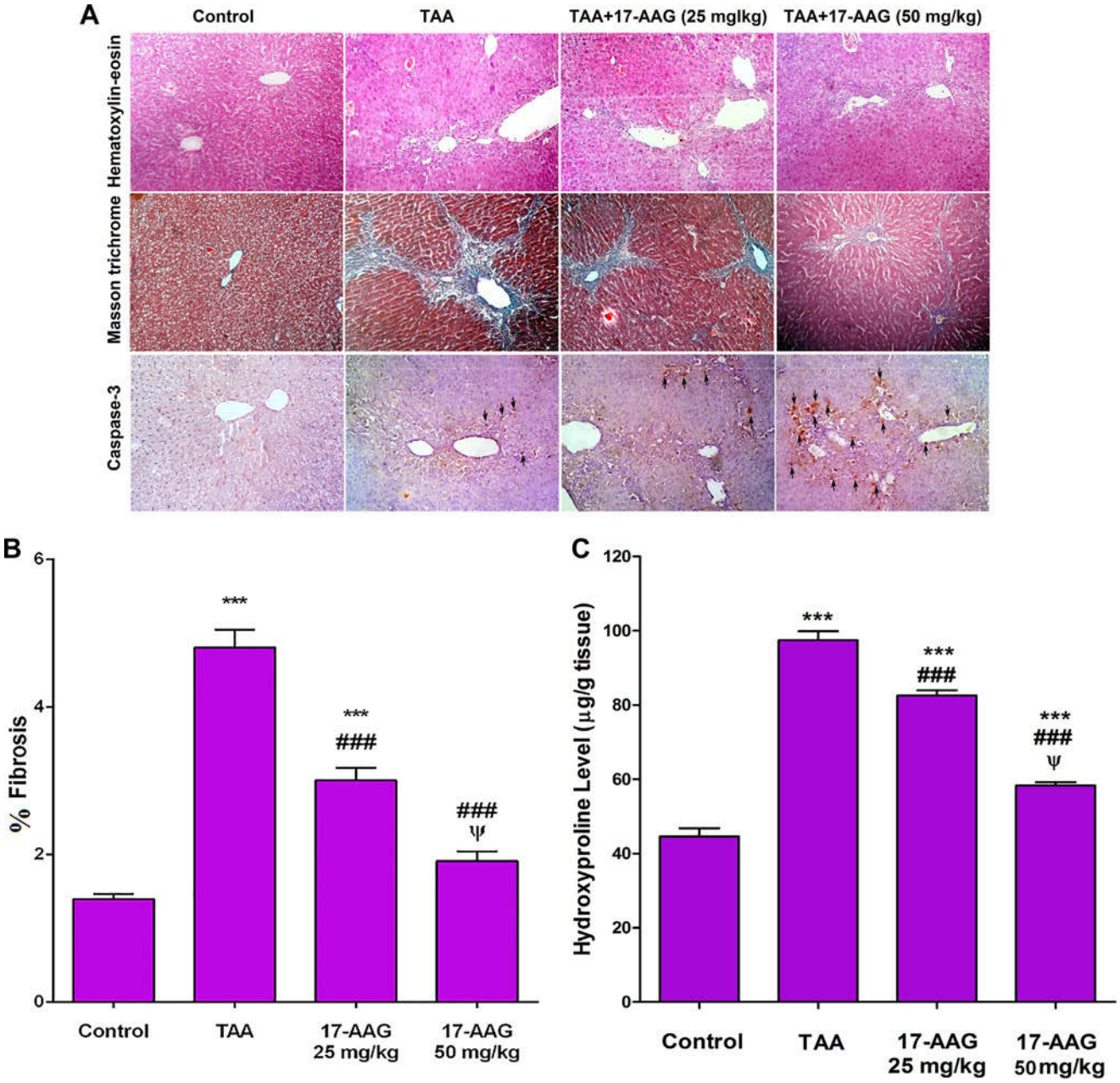

Fig. 1. (A) Representative images of liver sections [100X] showing effect of 17-AAG on inflammatory cells infiltration (*hematoxylin–eosin*), collagen deposition (*Masson trichrome*) and caspase-3 activity in thioacetamide (TAA) injected mice. (B) Effect on percentage (%) fibrosis. (C) Effect on hydroxyproline level. Significance ($n = 8$): ***, ### $p < 0.001$ compared to control and thioacetamide groups respectively, $^{\psi}$ $p < 0.5$ compared to 17-AAG (25 mg/kg) group.

causes similar alterations to those observed in alcoholic liver fibrogenesis [26]. Previously, liver fibrosis was considered as a passive irreversible process and now it is deemed as a wound-healing response to repeated liver injury [27]. Following liver injury by thioacetamide, release of pro-inflammatory and pro-fibrogenic cytokines, hepatocytes damage and quiescent HSCs activation take place [28].

The key step after injury involves the transformation and activation of HSCs into myofibroblast-like cells that express SMA [29] and produce extracellular matrix as collagen and glycoproteins [30]. Drastic matrix deposition with cross-linking of collagen and failure of activated HSCs to undergo apoptosis impair hepatocytes regeneration and limit resolution [31]. Many anti-fibrotic strategies that have been experimentally and clinically tested include removal of the inducible agent, reducing HSCs activation, inducing HSCs apoptosis, decreasing excessive extracellular matrix production and finally aiding in hepatocytes regeneration [32,33].

The present study was constructed to test the effect of the Hsp90 inhibitor 17-AAG on thioacetamide induced liver fibrosis and subsequently its possible application as an anti-fibrotic strategy. Effect of 17-AAG on fibrosis percentage, hepatocytes functionality, extracellular matrix production, HSCs activation and apoptosis was examined. Results showed the novel ability of Hsp90 inhibitor to attenuate thioacetamide induced liver injury *in vivo*.

Although some proteins that are expressed in response to cellular stresses may need Hsp90 for their stability and activity [34], Hsp90 role in liver fibrosis remains confounding. Some studies reported an increase in Hsp90 associating macrophage activation in liver disease [35]. Others suggest that Hsp90

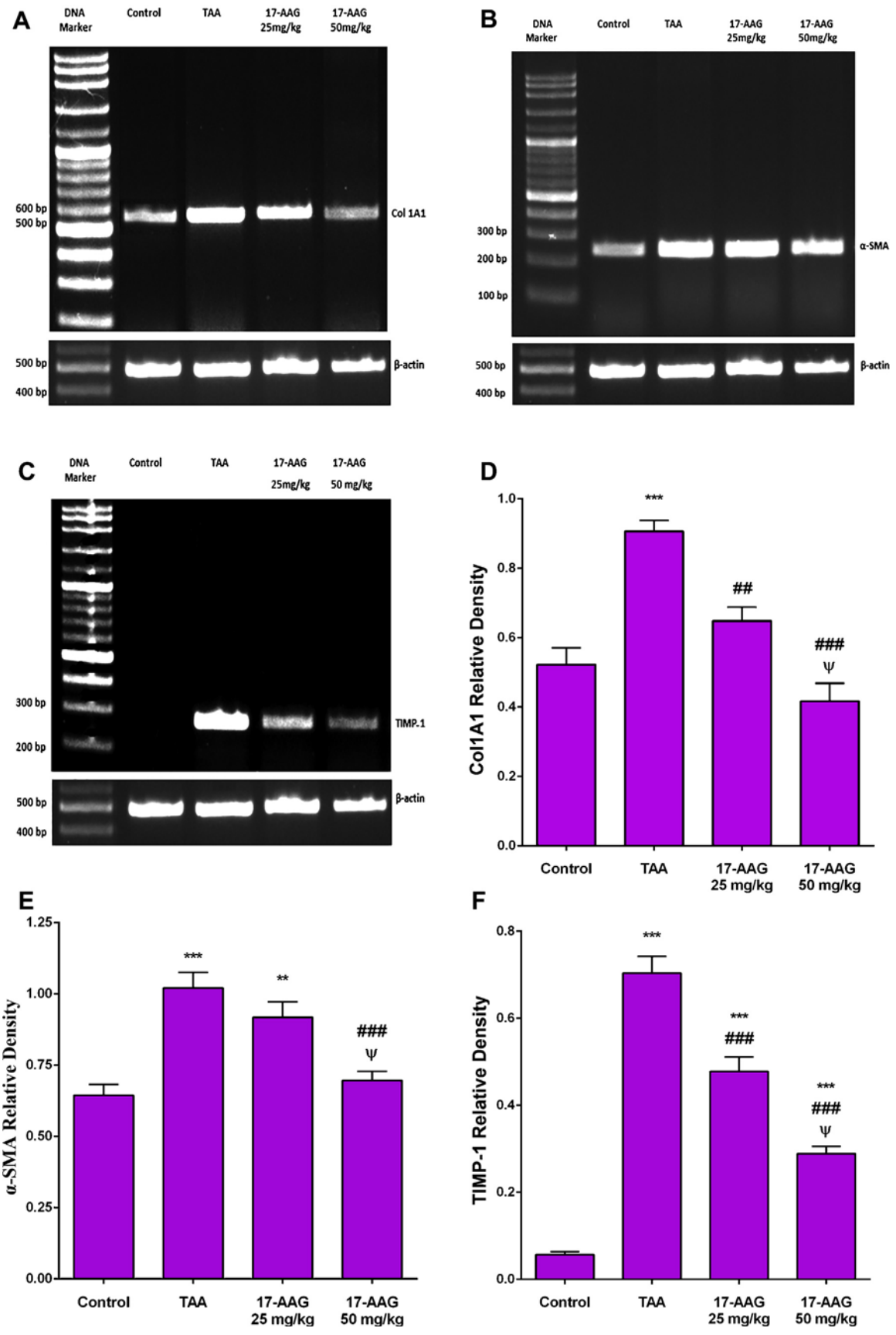

Fig. 2. Representative electropherograms showing RT-PCR separated mRNA bands of (A) collagen-1-alpha1 (Col1A1), (B) α-smooth muscle actin (α-SMA), (C) tissue inhibitor metalloproteinase-1 (TIMP-1); effect of 17-AAG (25, 50 mg/kg) injection on mRNA relative density of (D) Col1A1/β-actin, (E) α-SMA/β-actin, (F) TIMP-1/β-actin in thioacetamide (TAA) injected mice. Significance ($n = 8$): **, *** $p < 0.01$, 0.001 compared to control group, $^{\#\#}$, $^{\#\#\#}$ $p < 0.01$, 0.001 compared to TAA received group, $^{\psi}$ $p < 0.05$ compared to 17-AAG (25 mg/kg) received group.

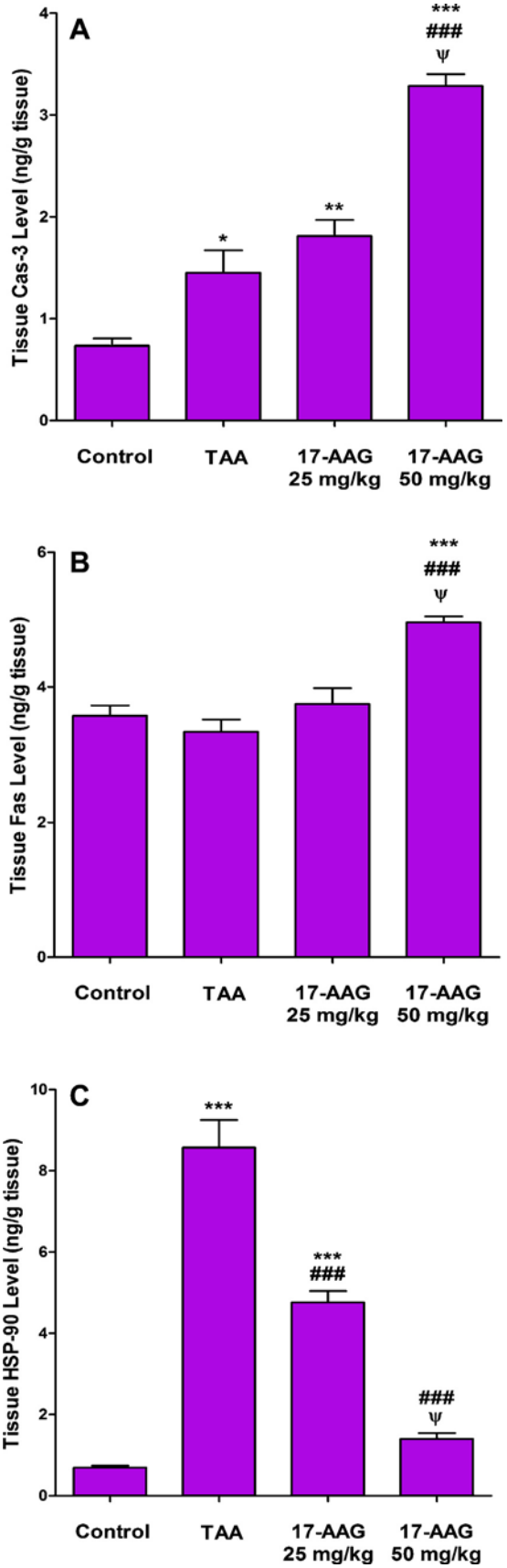

Fig. 3. Effect of 17-AAG (25, 50 mg/kg) on hepatic tissue level of (A) caspase-3 (Cas-3), (B) factor related apoptosis (Fas), (C) heat shock protein 90 (Hsp90) in thioacetamide (TAA) injected mice. Significance ($n = 8$): *, **, *** $p < 0.05$, 0.01, 0.001 compared to control group, ### $p < 0.001$ compared to thioacetamide received group, ψ $p < 0.05$ compared to 17-AAG (25 mg/kg) received group.

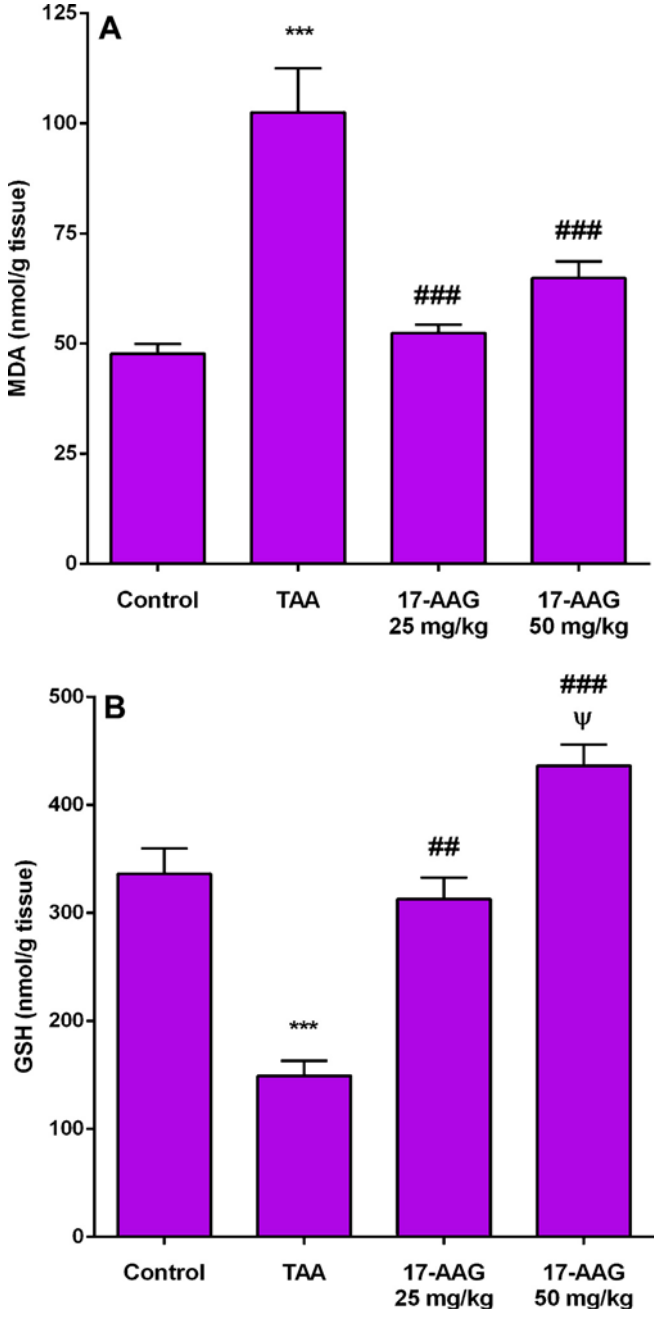

Fig. 4. Effect of 17-AAG (25, 50 mg/kg) on (A) malondialdehyde (MDA), (B) reduced glutathione (GSH) in thioacetamide (TAA) injected mice. Significance ($n = 8$): *** $p < 0.001$ compared to control group, ##, ### $p < 0.01$, 0.001 compared to thioacetamide received group, ψ $p < 0.05$ compared to 17-AAG (25 mg/kg) received group.

inhibition is beneficial in alleviating lipopolysaccharide mediated liver injury [16].

Histopathological examination applied to assess degree of fibrosis revealed that 17-AAG protected hepatocytes from thioacetamide induced injury. Col1A1 (collagen type I) as a major component of total collagen constituting the extracellular matrix released by activated HSCs is used as a marker for fibrosis [36]. Its over-expression by thioacetamide was lowered by 17-AAG with higher extent at high dose level an effect that can be explained by involvement of Hsp90 in metalloproteinases production pathways or in activation of HSCs. The latter effect can be ensured by downregulation of hepatic α-SMA mRNA expressed by the hepatic myofibroblastlike cells.

Our results showed that thioacetamide intoxication releases reactive oxygen species and suppress antioxidant mechanisms coinciding with previous findings [37]. 17-AAG was capable of reducing lipid peroxidation (low malondialdehyde levels) through restoring the antioxidant GSH hepatic capacity. Being a housekeeping chaperone released in response to stress, Hsp inhibition is suggested to have an impact on oxidative stress dependent fibrogenic pathways. And so, its inhibition can hold off IκB phosphorylation and release of NFκB a major participant in HSCs activation [38] and survival when it translocates into the nucleus [19].

In addition to reducing HSCs activation and balancing oxidative stress, 17-AAG can beneficially help in inducing HSCs apoptosis through a caspase pathway and allow hepatocytes regeneration. This is inferred by effect of 17-AAG on caspase-3 and Fas expression in liver tissue. Interestingly, TIMP-1 mRNA expression in liver was reduced by the tested inhibitor (especially at high dose). Many studies reported that TIMPs, mainly type 1, are the key regulators of metalloproteinases activity and matrix degradation promoting hepatic fibrosis through inhibiting matrix decomposition [39]. Reduced TIMP-1 level was also found to play a role in the regulation of hepatocyte regeneration where its lack leads to enhancing hepatocyte proliferation but its over-expression is associated with delayed proliferation [40].

Thus, reducing TIMP-1 levels may lead to restoring metalloproteinases activity causing degradation of matrix proteins and release of hepatocytes growth factor allowing hepatocytes expansion and their reentry into the cell cycle [41]. Tissue inhibitor metalloproteinase-1 is furthermore engaged in protecting HSCs from apoptosis *via* activating phosphatidyl inositol 3-kinase and ERKs resulting in down-regulation of caspases opposing fibrosis resolution. Therefore, regulating TIMP-1 expression by 17-AAG may trigger HSCs proapoptotic signals [42–44].

Conclusion

The Hsp90 inhibitor 17-AAG at certain dose level can attenuate liver fibrosis through (i) counterbalance of oxidative stress induced HSCs proliferation and hepatocytes injury, (ii) downregulation of TIMP-1 promoting matrix degradation, hepatocytes regeneration, (iii) induction of apoptosis through a caspase pathway.

Funding body

The study did not receive any fund from any organizations and was carried out only on the authors expenses

Conflict of interest

The authors have no conflict of interest to declare.

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