Verbenalin attenuates hepatic damage and mitochondrial dysfunction in alcohol-associated steatohepatitis by regulating MDMX/PPARα-mediated ferroptosis

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Abstract

Alcoholic steatohepatitis (ASH) is one of the predominant causes of liver-related morbidity and mortality worldwide. However, effective therapy for ASH is still lacking. Notably, increasing evidence indicates ferroptosis may counteract the injury of the ASH. We recently identified a novel strategy for attenuating ASH by an effective adjuvant via ferroptosis through targeting MDMX. Verbenalin, which is a major compound in Verbena officials L (Verbenaceae), is generally recognized as a kind of safe food by the U.S. Food and Drug Administration. In this study, we found for the first time that Verbenalin is protective against alcoholic liver injury through transmission electron microscopy, Fe2+ content detection and other research methods. Its effect can not only inhibit oxidative stress such as MDA, SOD, GSH, ROS, 4-HNE and other related indicators, but also inhibit the ferroptosis of hepatocytes caused by alcohol. It was further confirmed by computer docking that verbenalin was targeted to inhibit MDMX activity, promote PPAR-alpha activation, and inhibit alcohol-induced ferroptosis in hepatocytes. Hence, Verbenalin might be employed as a promising natural supplement for alcoholic liver injury drug therapy.

Introduction

Alcoholic steatohepatitis (ASH) is one of the predominant causes of serious liver-related diseases because of long-term alcohol consumption, resulting in high morbidity and mortality worldwide(Gao & Bataller, 2011; Seitz et al., 2018; Xiao et al., 2019). If not treated effectively or on time, ASH may lead to fibrosis, and cirrhosis and may even develop into hepatic carcinoma. Much work has been done in recent years to provide insight into the pathogenesis of ASH (Seitz et al., 2018). However, valid therapeutic drugs are still lacking.

Clinical studies show that ASH patients frequently exhibit a wide range of clinical manifestations associated with iron overload and iron homeostasis, suggesting that ferroptosis is involved in an alcoholic liver injury(Sui, Jiang, Chen, Yang & Zhu, 2018; Sun et al., 2020). Ferroptosis is a novel form of regulated cell death (RCD) characterized by the overwhelming iron dependent accumulation of lipid hydro peroxides(Wu et al., 2021a). Ferroptosis plays a significant role in multiple kinds of organic diseases, such as brain, heart, kidney, and liver diseases(Li et al., 2020). Kohgo et al reported high mortality from alcoholic cirrhosis is associated with oxidative damage caused by iron overload(Kohgo, Ikuta, Ohtake, Torimoto & Kato, 2007). Kong et al were also reported hepatic fibrosis is also associated with oxidative injury and ferroptosis(Kubica, Szopa, Dominiak, Luczkiewicz & Ekiert, 2020). Thus, triggering or inhibiting ferroptosis might provide novel therapeutic strategy for treating liver diseases.

Verbena officials L. (Verbenaceae), commonly known as vervain, is a widespread perennial herb that played a traditional role in empirical medicine over centuries(Kubica, Szopa, Dominiak, Luczkiewicz & Ekiert, 2020).

Vervain is "generally recognized as safe" (GRAS) as a food by the U.S. Food and Drug Administration(Cao, Miao, Qiao, Bai & Li, 2018). Today it is listed in the European, British, and Chinese Pharmacopoeias respectively and is mainly used because of its expectorant, anti-rheumatic, and diuretic effects(Cao, Miao, Qiao, Bai & Li, 2018). Studies(Calvo, 2006; Cao, Miao, Qiao, Bai & Li, 2018; Deepak & Handa, 2000) have confirmed that verbenalin, the main component of verbena, has antioxidant effects, but its role in alcoholic liver injury is not explicit. In this study, we firstly reported that Verbenalin ameliorates alcoholic liver injury by suppressing MDMX-mediated ferroptosis.

Materials and methods

Materials and reagents

Verbenalin was provided from Meilune Biology Technology (Ruike Biotechnology CAS drk-0085, ChengDu, China). MDMX inhibitor NSC207895 was obtained from Calbiochem, PPARα agonist (Pirinixic acid) was obtained from Selleckchem, PPARα antagonist (GW6471) was obtained from Santa Cruz Biotechnology, β-actin, MDMX primary antibodies and goat anti-rabbit or anti-mouse immunoglobulin G (IgG) horseradish peroxidase (HRP) secondary antibodies were purchased from Bioss (Beijing, China). Enzyme linked immunosorbent assay (ELISA) kits were purchased from Elabscience Biotechnology Co. Ltd. (Wuhan, China), and the ALT (C009-2), AST (C010-2), TG (A110-1), GSH (A006-2-1), MDA (A003-1), SOD (A001-3) assay kits were purchased from Jiancheng Biology Institution PeproTech (Nanjing, Jiangsu, China). Cell Counting Kit-8(CCK-8) (BB-4202) were obtained from BestBio (Shanghai, China).

Cell culture

AML-12 cells were provided by the Chinese Academy of Sciences (Shanghai, China), maintained in F-12 (HyClone, USA) supplemented with 10% foetal bovine serum (FBS, Merck Millipore, Darmstadt, Germany), and incubated at 37°C in an atmosphere of 5% CO_2. Meanwhile, Then cells were cultured by adding 200 mM ethanol for 24 h or incubation with 100 μM Verbenalin in the presence of 200 mM ethanol for 24 h.

CETSA

The cellular target identification of Verbenalin was performed using CETSA. Briefly, the cells were treated with or without Verbenalin for 24 h and then heated individually at different temperatures (37-64 °C) for 3 min followed by cooling on ice. The cell lysates were obtained by five repeated freeze-thaw cycles in liquid nitrogen. The soluble lysates were centrifuged, and supernatants were detected by a western blot(Wan et al., 2020).

Cytotoxicity assay

The CCK-8 assay kit was used to test cytotoxicity in vitro. AML-12 cells were plated in 96-well plates at a density of 5,000 cells per well. AML-12 cells were separately incubated with ethanol and Verbenalin for 24h. Then, a CCK-8 solution diluted 1/10 with 5% FBS F-12, was added to each well and incubated for 2h at 37degC. The optical density (OD) was measured at 450 nm. The percent of viable cells was calculated according to the formula.

Animal treatment

Male C57BL/6J mice weighing 18–20 g (6-8 weeks) were purchased from the Laboratory Animal Center of Anhui Medical University. All animal experiments were approved by the Institutional Animal Experimentation Ethics Committee of Anhui Medical University. All mice were housed in a comfortable environment and were adapted for 5 days before the experiment. All experimental procedures were reviewed and performed in accordance with the Animal Experiments Guidelines and Animal Care of the Chinese Academy of Sciences. A total of 36 mice were randomly divided into six groups of 6 animals, respectively control diet (CD)-fed mice, EtOH-fed mice, verbenalin-treated mice at the dose of 12.5, 25, 50 mg/kg/day and control diet (CD)-fed mice adds to 50 mg/kg/day verbenalin-treated. The modeling process of the Gao-binge protocol contains a total of 16 days, including a controlled liquid diet for the adaptive stage (3 days), a building period (13 days), alcohol gavage (single occurrence), and animal sample collection (1 day). The model group mice were

fed containing 5% ethanol liquid diets adding some vitamin and choline for 16 days, and mice were gavaged with a single binge ethanol administration (5 g/kg, body weight, 20% ethanol) at last day. Meanwhile, the verbenalin-treated mice were not only plus the ethanol administration, but also plus the medicines by gavage daily, whereas the CD-fed and CD-fed adds to 50 mg/kg/day verbenalin-treated mice were fed with control liquid diets and administered isocaloric maltose-dextrin by gavage on the final day. All diets were freshly prepared every day. The mice were killed 9 h after alcohol gavage, liver tissues and blood were collected for further investigation(Li et al., 2017; Meng et al., 2019).

ALT/AST/TG/MDA/GSH/SOD activity assay

Blood samples were collected for ALT,AST,TG,MDA,GSH and SOD measurements according to the manufacturer's instructions.

Histopathology

Liver tissues of mice were immersed in 10% formaldehyde (pH 7.4) fixative for 24 h and then embedded in paraffin. The sections (5μm thick) were stained with the H&E and oil red O. All experimental procedures were as followed standard program. The sections were observed and imaged using light microscopy.

Western blot analysis

AML-12 cells were extracted with RIPA lysis buffer (Beyotime, Shanghai, China) containing 1% PMSF. The protein samples were separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Nonspecific protein binding was blocked by incubating the membrane in TBST containing 5% skim milk at room temperature for 2 h and washed three times with TBST. Subsequently, the PVDF membranes were incubated for 24 h at 4 with primary antibodies (Beyotime, China) against β-actin (1:500), MDMX (1:500) were used to combine with the specific proteins. Horseradish peroxidase conjugated anti-rabbit and anti-mouse antibodies (1:5000) for 1 h at room temperature and washed three times with TBST. The proteins were visualized with an ECL-chemiluminescent kit (ECL-plus, Thermo Scientific, Pittsburgh, PA, USA).

Cytokine assay by ELISA

Blood samples were collected and centrifuged at 3000 g for 15 min to isolate the serum. The activity of MDMX, PPARα, ROS, 4-HNE and Iron Assay were detected by ELISA kits. The culture supernatants of AML-12 cells were measured using MDMX, PPARα, ROS, 4-HNE and Iron Assay ELISA kits.

Transmission electron microscopy

Cells and tissues were harvested and fixed with a PBS solution containing 2.5% glutaraldehyde for 24 h. After being washed in PBS, washed with phosphate buffer, fixed in osmium acid, and stained with uranyl acetate. After dehydration and embedding, samples were incubated in a 60 °C oven for 24 h. Digital images were acquired using a transmission electron microscope(Badgley et al., 2020).

Statistical analysis

The data were completed with GraphPad Prism 5.0 software (San Diego,CA).All in vitro experiments were repeated at least three times and all of the results were repeated at least six times in vivo. Two sets of date were analyzed by t -test, multiple-group comparisons were analyzed using the Kruskal-Wallis one-way ANOVA (ANOVA). Values of the $p < 0.05$ be statistically significant.

Results

Verbenalin alleviates hepatic injury in alcohol-associated steatohepatitis

To investigate the effects of Verbenalin (molecular structural formula, Figure 1a) on alcoholic steatohepatitis (ASH), we treated C57BL/6 mice with alcohol followed by the Gao-Binge protocol (Figure 1b). Histopathological change was a direct indication of liver injury. This study used both morphological changes in the appearance of liver and hepatocyte lesions as indicators to evaluate liver injury. The macroscopic appearance

showed verbenalin can reduce alcohol-induced liver damage. H&E staining showed that liver tissues in AH mice exhibited fat vacuoles, liver cell cord derangement, intercellular spaces dilatation and inflammatory cell infiltration, while verbenalin treatment significantly improved liver tissue injury in the dose-dependent manner (12.5, 25, 50 mg/kg) (Figure 1c). Similar results were shown in the serum levels of ALT and AST (Figure 1d and 1e). To determine the effect of verbenalin on lipid homeostasis, hepatic steatosis developed in the model of AH was assessed. Oil red O-stained sections showed the number of lipid droplet was significantly decreased in liver tissue after Verbenalin administration (Figure 1f). The levels of serum and tissues TG were also remarkably decreased in verbenalin-treated mice (Figure 1g and 1h). And similar to the above, all of these changes were dose-dependent, and the group treated with 50 mg/kg verbenalin showing the greatest effects. In addition, vehicle mice with verbenalin (50 mg/kg) did not cause observable damage.

To further study the beneficial of verbenalin in AH, we treated the AML-12 cell line with ethanol to establish an in vitro model. We treated the cells with different doses of ethanol and verbenalin for 24 h and measured the effects using an CCK8 assay (Figure S1). From these results, we found the beneficial effects of verbenalin were concentration-dependent over the range of 100 - 800 μM. Thus, in subsequent work, we used verbenalin at 100 μM (Figure 1i). As shown in figure 1j-1l, verbenalin suppressed ethanol-induced increases of ALT, AST and TG in AML-12 cells.

Verbenalin suppresses oxidative stress-induced ferroptosis in alcohol-associated steatohepatitis

Since verbenalin regulated lipid homeostasis, we analyzed whether the levels of oxidative stress-induced lipid peroxidation were suppressed by treatment with verbenalin in AH. Oxidative stress is characterized by the over-production of reactive oxygen species (ROS), we measured the level of ROS in serum and liver tissues. Compared with the vehicle group, the level of ROS production was elevated by alcohol and reduced by treatment with verbenalin (Figure 2a and 2b). Consistently, the intensity of 4-hydroxynonenal (4-HNE), a marker of oxidative stress-induced lipid peroxidation and cytotoxicity, and malondialdehyde (MDA), a final product of lipid peroxidation during oxidative stress, were measured. As shown in figure 2c - 2f, verbenalin decreased ethanol-induced increases of 4-HNE and MDA. We also analyzed the status of glutathione (GSH) and Superoxide Dismutase (SOD) as an indicator of oxidative stress. Verbenalin elevated the levels of GSH and SOD relative to AH (Figure 2g - 2j). Interestingly, similar to in vivo data, treatment with verbenalin suppressed the levels of ROS, 4-HNE and MDA, and elevated the levels of GSH and SOD in AML-12 cells (Figure 2k).

To investigate the molecular mechanism by which verbenalin regulates oxidative stress-induced hepatic damage, we examined whether verbenalin could suppress ferroptosis by oxidative stress-induced lipid peroxidation in AH. As shown in Figure 2i, the alcohol led to more serious mitochondrial swelling and vanished cristie in the livers of AH, while verbenalin could protect mitochondrial structure by transmission electron microscopy analysis. Further, we hypothesized that alcohol increases sensitivity to ferroptosis by upregulating Fe^{2+} and lipid peroxidation levels in cells. Thus, we measured the level of Fe^{2+} in serum and liver tissues. In support of our hypothesis, we found that alcohol increased the level of $Fe^{2+}$and the effect was reversed by verbenalin (Figure 2m and 2n). Similar results were also found in the AML-12 cells (Figure 2o and 2p). These results suggested that verbenalin suppressed oxidative stress-induced ferroptosis in alcohol-associated steatohepatitis.

Verbenalin targets MDMX toinhibit oxidative stress and ferroptosis in vitro

To further define the molecular mechanism of verbenalin, molecular docking was used to search for the target of verbenalin. The CDOCKER docking results showed that verbenalin was located in catalytic center of MDMX (Figure 3a), and the tetrahydropyran ring in this molecule formed two conventional hydrogen bonds with the amino acid LEU. Moreover, the ester group in the molecule establishes conventional hydrogen bond interactions with amino acid TYR67 and with amino acid HIS73, respectively. The amino acid GLU69 formed two hydrogen bond interactions with two hydrogen atoms in the molecule linked to the esteryl carbon, and the methyl group on cyclopentane had hydrophobic interactions with the amino acid HIS96.

In order to inspect verbenalin influence on MDMX activity, the activity of MDMX in serum and liver

tissues was detected by ELISA. It was interesting to explore that the activity of MDMX was significantly blunted by verbenalin in serum and liver tissues in different group of mice (Figure 3b and 3c). Similar in vitro results were obtained in AML-12 cells (Figure 3d). To determine the effect of verbenalin on MDMX stability, we performed cellular thermal shift assay (CETSA) and found that verbenalin increased MDMX thermotolerance in a temperature gradient (37–64 *C) (Figure 3e).

To investigate whether downregulated MDMX was directly involved in the suppression of ferroptosis, we measured the levels of oxidative stress makers by MDMX inhibitor NCS207895 in AML-12 cells (Figure 3f and 3g). As shown in Figure 3h, treatment with NCS207895 suppressed the levels of ROS, 4-HNE and MDA, and elevated the levels of GSH and SOD in AML-12 cells. Changes in the mitochondrial morphology were observed by electron microscopy (Figure 3i). We observed decreased mitochondrial swelling, cristae disorder, and matrix particles with the treatment of NCS207895. In addition, the content of Fe^{2+} was reduced with the treatment of NCS207895 (Figure 3j).

To determine unambiguously whether verbenalin targets MDMX to inhibit oxidative stress and ferroptosis, MDMX was overexpressed, via lentivirus transfection in AML-12 cells (Figure 4a). Then, we treated ethanol-induced AML-12 cells with pEGFP-C1/MDMX and verbenalin (100μM) for 24h (Figure 4b). ELISA results showed that overexpression of MDMX in AML-12 cells elevated the levels of ROS, 4-HNE and MDA, and suppressed the levels of GSH and SOD, while the role of antioxidant was suppressed with the treatment of verbenalin (Figure 4c). Furthermore, the transmission electron microscopy showed increased mitochondrial dysfunction in alcohol-induced pEGFP-C1/MDMX- transfected AML-12 cells were not blunted by verbenalin (Figure 4d). Similar observation was further confirmed by the level of Fe^{2+} (Figure 4e). Therefore, MDMX may be the target of verbenalin when this compound has its beneficial effects.

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To determine the molecular mechanism of MDMX, co-immunoprecipitation (Co-IP) assay was performed in AML-12 cells. The results showed that PPARα was pulled-down by MDMX (Figure 5a). The activity of PPARα was significantly upregulated with the treatment of NCS207895, MDMX inhibitor, while was downregulated with pEGFP-C1/MDMX (Figure 5b and 5c).

In fact, the PPAR family of transcription factors are known to be involved in large-scale rewiring of lipid homeostasis, we hypothesized that this effect of MDMX on ferroptosis might be mediated by PPARα. To test this, we evaluated whether altering PPARα activity altered the sensitivity of cells to ferroptosis. To this end, we used PPARα-specific agonist (pirinixic acid) and a PPARα antagonist (GW6471). We confirmed the effectiveness of these compounds by testing the effect on the levels of oxidative stress makers (Figure 5d). As shown in Figure 5e, treatment with pirinixic acid suppressed the levels of ROS, 4-HNE and MDA, and elevated the levels of GSH and SOD in AML-12 cells. Meanwhile, electron microscopy showed that PPARα-specific agonist suppressed ferroptosis (Figure 5f), and downregulated the level of Fe^{2+}(Figure 5g).

Next, we assayed the protective effect of MDMX inhibitor against oxidative stress-induced ferroptosis with or without the treatment of PPARα antagonist in the AML-12 cells (Figure 6a). Interestingly, the effect of NCS207895 in downregulating the levels of ROS, 4-HNE and MDA, and upregulating the levels of GSH and SOD were blocked by GW6471 in AML-12 cells (Figure 6b). Furthermore, electron microscopy showed that the protective effect of NCS207895 against ferroptosis, was suppressed in GW6471 treated AML-12 cells (Figure 6c). Similarly, the observation was further confirmed by the level of Fe^{2+}(Figure 6d). These results suggested that PPARα activity plays a key role in facilitating the abilities of MDMX to promote ferroptosis.

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In order to inspect the influence of verbenalin on PPARα activity, the activity of PPARα in serum and liver tissues was detected by ELISA. It was interesting to explore that the activity of PPARα was significantly increased by verbenalin in serum and liver tissues in different group of mice (Figure 7a and 7b). Similar in vitro results were obtained in AML-12 cells (Figure 7c).

Furthermore, we assayed the protective effect of verbenalin against oxidative stress-induced ferroptosis with or without the treatment of PPARα antagonist in the AML-12 cells (Figure 7d). As shown in figure 7e, the effect of verbenalin in downregulating the levels of ROS, 4-HNE and MDA, and upregulating the levels of GSH and SOD were blocked by GW6471 in AML-12 cells. Consistently, electron microscopy showed that the protective effect of verbenalin against ferroptosis, was suppressed in GW6471 treated AML-12 cells (Figure 7f). Similarly, the observation was further confirmed by the level of Fe^{2+} (Figure 7g). These results suggested that verbenalin regulates MDMX/PPARα-mediated ferroptosis in AML-12 cells.

Discussion

The present study demonstrated, for the first time, that verbenalin alleviated hepatic injury and regulated lipid homeostasis in a mouse model of steatohepatitis induced by binge ethanol feeding. Emerging evidenced had demonstrated alcoholic steatohepatitis (ASH) is a leading cause of liver disease worldwide(Louvet & Mathurin, 2015). Therefore, it is necessary to find effective drug to attenuate ASH. Natural products often play an important role in clinical therapy. Verbenalin is the major active component of vervain, which is recognized as a safe food by the U.S. Food and Drug Administration. On the basis of modern pharmacological and clinical studies, it is shown that verbenalin possesses various biological actions(Cao, Miao, Qiao, Bai & Li, 2018; Deepak & Handa, 2000). In this study, verbenalin could ameliorate ASH as supported by results of ALT, AST and TG analysis, the appearances of the liver and histological observations.

A growing body of evidence suggests that ferroptosis may serve as a promising target for the prevention and treatment of ASH. Ferroptosis, an iron-dependent form of nonapoptotic cell death(Li et al., 2020), occurs through an increase in cellular phospholipid peroxidation in the context of a compromised phospholipid peroxide repair system(Wang et al., 2021; Wu et al., 2021b). With respect to ASH, liver cells are more susceptible to ferroptosis. Alcoholic-injured hepatocytes exhibit characteristic morphological changes in ferroptosis, including condensed mitochondrial membrane densities and a reduced volume, iron overload in mitochondria, accumulated mitochondrial ROS(Imai, Matsuoka, Kumagai, Sakamoto & Koumura, 2017). Therefore, we measured changes of the levels of oxidative stress-induced peroxidation in both in vivo and in vitro experimental ASH models. The increase of oxidative stress resulted in mitochondrial dysfunction and ferroptosis. Treatment with verbenalin dramatically attenuated mitochondria dysfunction and ferroptosis in vivo and in vitro. Therefore, verbenalin may act as an effective treatment for ASH by modulating mitochondrial dysfunction.

Results obtained with molecular docking, murine double minute X (MDMX) activity, and CETSA, have revealed that verbenalin can regulate ferroptosis in a MDMX-dependent manner. The oncogene MDMX, also known as MDM4 is a critical negative regulator of the tumor suppressor p53(Dobbelstein & Levine, 2020; Wang et al., 2020) and has been implicated in the initiation and progression of human cancers(Skalniak, Surmiak & Holak, 2019; Yu, Xu, Mo, Yuan, Cheng & Qin, 2020). Increasing evidence (Yu, Xu, Mo, Yuan, Cheng & Qin, 2020)indicates that MDMX is often amplified and highly expressed in human cancers, promotes cancer cell growth, and inhibits apoptosis(Venkatesh et al., 2020). Therefore, MDMX is becoming one of the most promising molecular targets for developing anticancer therapeutics. Venkatesh et al (Venkatesh et al., 2020) reported MDMX control ferroptosis sensitivity through their ability to regulate lipid homeostasis. Consistent with prior reports, we found MDMX inhibitor not only suppressed the reduction of oxidative stress and lipid peroxidation resulting from alcoholic treatment, but enhanced it further beyond the levels of the vehicle control due to increased levels of reduced GSH and SOD. This finding is in line with our hypothesis of large-scale rewiring of lipid metabolism caused by MDMX in ASH. Furthermore, our data show that MDMX control ferroptosis sensitivity through their ability to regulate lipid homeostasis. Our findings with MDMX overexpression suggest that verbenalin hepatoprotective mechanisms might be largely contributed by modulating mitochondria dysfunction and ferroptosis which related to targeting MDMX.

The Co-IP results suggested that there may be direct interactions between MDMX and PPARα, and we found that, in AML-12 cell lines, MDMX behave like PPARα antagonists. We then evaluated the role of PPARα, a transcription factor whose activity has been previously reported to be regulated by MDM2 under some conditions (Venkatesh et al., 2020). The PPAR family of receptors, which are master regulators of

cellular metabolism have been implicated in several diseases(Gervois, Torra, Fruchart & Staels, 2000; Lu et al., 2019; Rosenson, 2007). In fact, the PPAR family are known to be involved in large-scale rewiring of lipid homeostasis, particularly in response to stress(Auwerx, Schoonjans, Fruchart & Staels, 1996; Rosenson, 2007). Pharmacological fibrates are frequently used as triglyceride-lowering drugs that function by enhancing PPARα activity(Kersten, 2008; van Raalte, Li, Pritchard & Wasan, 2004). In accordance with these previous reports, our data demonstrated that PPARα agonist was dramatically attenuated mitochondria dysfunction and ferroptosis in vitro. We hypothesized that this effect of MDMX on ferroptosis might be mediated by PPARα. To test our hypothesis, MDMX and PPARα were silenced, via the inhibitors in AML-12 cells. We collected the samples and used the same method as described previously to conduct various tests and analyses. As expected, low levels of MDMX alleviates alcohol-induced mitochondria dysfunction and ferroptosis in AML-12 cells, which was relieved by PPARα antagonist. Moreover, by comparing the effects of verbenalin under the state of PPARα antagonist, the antagonist of PPARα suppressed the effects of verbenalin on alcohol-induced hepatocyte injury, which indicated that PPARα was tightly related to the function of verbenalin in inhibiting lipid accumulation, mitochondria dysfunction and ferroptosis. We have concluded that verbenalin could act as an MDMX antagonist to up-regulate PPARα to inhibit the progression of ASH.

In conclusion, we have demonstrated that verbenalin prevented hepatic TG accumulation in ASH mice and in the AML-12 cell line treated with alcohol, and alleviated mitochondria dysfunction and ferroptosis (Figure 8). Verbenalin decreased the severity of experimental alcohol steatohepatitis via mechanisms likely to involve the regulation of MDMX/PPARα. Our studies confirmed that verbenalin may become a promising drug to treat or relieve ASH.

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Credit Author Statement

Jiahui Dong performed most of the experiments, analyzed the data and wrote the manuscript. Taotao Ma, Cheng Huang and Jun Li supervised the research and edited the manuscript. Zeng Li, Changlin Du and Zhonghao Wang provided a series of experimental instructions; Fanle Zeng, Zhenzhen Qian, Chuangting Xu and Qi Wang helped the experiments. Lei Zhang and Xionwen Lv revised the manuscript. All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

Declaration of Competing Interest

The authors report no declarations of interest.

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Figure legends

FIGURE 1 Verbenalin (VE) alleviates hepatic injury in alcohol-associated steatohepatitis (ASH)

1. Molecular structural formula of verbenalin.
2. Experimental flow chart.
3. Representative macroscopic appearances of the livers and H&E staining (×200).
4. Serum ALT level;
5. Serum AST level;
6. Oil Red O staining (×200) of livers.
7. Serum TG level;
8. Tissue TG level;
9. AML-12 cells were exposed to Ethanol (200mM) and VE (100μM) for 24 hours.
10. The ALT level in AML-12 cells treated by ethanol and verbenalin;
11. The AST level in AML-12 cells treated by ethanol and verbenalin;
12. The TG level in AML-12 cells treated by ethanol and verbenalin;

Data shown are means ± SEM; n [?] 5 in each group.

FIGURE 2 Verbenalin (VE) suppresses oxidative stress-induced ferroptosis in alcohol-associated steatohepatitis (ASH)

1. Serum ROS level;
2. Tissue ROS level;
3. Serum 4-HNE level;
4. Tissue 4-HNE level;
5. Serum MDA level;
6. Tissue MDA level;
7. Serum GSH level;

8. Tissue GSH level;
9. Serum SOD level;
10. Tissue SOD level;

The levels of ROS, 4-HNE, MDA, GSH, SOD in AML-12 cells treated by ethanol and verbenalin;

1. Electron microscopy (x10000; x20000) in tissues;
2. The Fe^{2+} level in serum ;
3. The Fe^{2+} level in tissue;

Electron microscopy (x6700; x13500) in AML-12 cells treated by ethanol and verbenalin;

The Fe^{2+} level in AML-12 cells treated by ethanol and verbenalin;

Data shown are means +- SEM; n [?] 5 in each group.

FIGURE 3 Verbenalin (VE) targets MDMX to inhibit oxidative stress and ferroptosis in vitro

1. verbenalin targeted to MDMX;
2. Serum MDMX activity;
3. Tissue MDMX activity;
4. The MDMX activity in AML-12 cells treated by ethanol and verbenalin;;
5. MDMX to different temperature gradients;
6. AML-12 cells were exposed to Ethanol (200mM) and NCS207895 (5μM) for 24 hours.
7. Protein levels of MDMX;
8. The levels of ROS, 4-HNE, MDA, GSH, SOD in AML-12 cells treated by ethanol and NCS207895;
9. Electron microscopy (×6700; ×13500) in AML-12 cells treated by ethanol and NCS207895;
10. The Fe^{2+} level in AML-12 cells treated by ethanol and NCS207895;

Data shown are means ± SEM; n [?] 5 in each group.

FIGURE 4 The protective effect of verbenalin (VE) was decreased by MDMX up-regulation.

1. Protein levels of MDMX;
2. AML-12 cells were exposed to Ethanol (200mM), VE(100μM) and pEGFP-C1/MDMX for 24 hours.
3. The levels of ROS, 4-HNE, MDA, GSH, SOD in AML-12 treated by ethanol, verbenalin and pEGFP-C1/MDMX;
4. Electron microscopy (×6700; ×13500) in AML-12 treated by ethanol, verbenalin and pEGFP-C1/MDMX;
5. The Fe^{2+} level in AML-12 treated by ethanol, verbenalin and pEGFP-C1/MDMX;

Data shown are means ± SEM; n [?] 5 in each group.

FIGURE 5 PPARα activity plays a key role in facilitating the abilities of MDMX to promote ferroptosis

1. The interaction between MDMX and PPARα.
2. PPARα activity in AML-12 cells treated by MDMX inhibitor;
3. PPARα activity in transfected AML-12 cells;
4. AML-12 cells were exposed to Ethanol (200mM) and pirinixic acid (10μM) for 24 hours.
5. The levels of AML-12 ROS, 4-HNE, MDA, GSH, SOD in AML-12 cells treated by pirinixic acid;
6. Electron microscopy (×6700; ×13500) in AML-12 cells treated by pirinixic acid;
7. The Fe^{2+} level in AML-12 cells treated by pirinixic acid;

Data shown are means ± SEM; n [?] 5 in each group.

FIGURE 6 MDMX alter the antioxidant responses of cells through PPARα activity

1. AML-12 cells were exposed to Ethanol (200mM), GW6471 (10μM) and NCS207895 (5μM) for 24 hours;

2. The levels of ROS, 4-HNE, MDA, GSH, SOD in AML-12 cells treated by Ethanol, GW6471 and NCS207895;
3. Electron microscopy (×6700; ×13500) in AML-12 cells treated by ethanol, GW6471 and NCS207895;;
4. The Fe^{2+} level in AML-12 cells treated by ethanol, GW6471 and NCS207895;;

Data shown are means ± SEM; n [?] 5 in each group.

FIGURE 7 Verbenalin (VE) regulates MDMX/PPARα-mediated ferroptosis in AML-12 cells

1. Serum PPARα activity;
2. Tissue PPARα activity;
3. AML-12 cells PPARα activity;
4. AML-12 cells were exposed to Ethanol (200mM), GW6471 (10μM) and VE(100μM) for 24 hours;
5. The levels of ROS, 4-HNE, MDA, GSH, SOD in AML-12 cells treated by ethanol, GW6471 and verbenalin;
6. Electron microscopy (×6700; ×13500) in AML-12 cells treated by ethanol, GW6471 and verbenalin;
7. The Fe^{2+} level in AML-12 cells treated by ethanol, GW6471 and verbenalin;

Data shown are means ± SEM; n [?] 5 in each group.

FIGURE 8 Verbenalin (VE) attenuates hepatic damage and mitochondrial dysfunction in alcohol-associated steatohepatitis by regulating MDMX/PPARα-mediated ferroptosis

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