Demethyleneberberine, a natural mitochondria-targeted antioxidant, inhibits mitochondrial dysfunction, oxidative stress and steatosis in alcoholic liver disease mouse model

Pengcheng Zhang, Xiaoyan Qiang, Miao Zhang, Dongshen Ma, Zheng Zhao, Cuisong Zhou, Xie Liu, Ruiyan Li, Huan Chen, Yubin Zhang

State Key Laboratory of Natural Medicines, Department of Biochemistry,
China Pharmaceutical University, Nanjing 210009, China

Institute of Toxicology, Jiangsu Provincial Center for Disease Prevention and Control,
Nanjing 210009, China
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Corresponding: Yubin Zhang,
Department of Biochemistry, China Pharmaceutical University,
24 Tongjia Xiang, Nanjing 210009, China.
Tel.: 86-25-83271300.
Email: ybzhang@cpu.edu.cn.

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Nonstandard Abbreviations: DMB, demethyleneberberine; ROS, reactive oxygen species; RNS, reactive nitrogen species; CYP2E1, cytochrome P450 2E1; iNOS, inducible nitric oxide synthase; HIF1α, hypoxia inducible factor α; ALD, alcoholic liver disease; SIRT 1, sirtuin 1; AMPK, adenosine 5'-monophosphate (AMP)-activated protein kinase; PGC-1α, peroxisome proliferator-activated receptor-gamma Co-activator-1α; MMP, mitochondrial membrane potential; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DCFH-DA, dihydriclorofluorescein diacetate; TBARS, thiobarbituric acid reactive substances; GSH, glutathione; GPx, glutathione peroxidase; FCM, flow cytometry; LD Diet, Lieber-DeCarli liquid diet; DAB, diaminobenzidine; OG, orally gavage; IP, intraperitoneal; IV, intravenous; IHC, immunohistochemistry
ABSTRACT

Excessive alcohol consumption induces oxidative stress and lipid accumulation in liver.

Mitochondria have long been recognized as the key target for alcoholic liver disease (ALD).

Recently artificial mitochondria-targeted antioxidant MitoQ has been used to treat ALD effectively in mice. Here we introduce a natural mitochondria-targeted antioxidant Demethyleneberberine (DMB) which has been found in Chinese herb *Cortex Phellodendri chinensis*. The protective effect of DMB on ALD was evaluated with HepG 2 cells and acutely/chronically ethanol-fed mice, mimicking two common patterns of drinking in human.

The results showed that DMB, which is composed of a potential antioxidant structure, could penetrate the membrane of mitochondria and accumulate in mitochondria either in *vitro* or *vivo*. Consequently, the acute drinking-caused oxidative stress and mitochondrial dysfunction were significantly ameliorated by DMB. Moreover, we also found that DMB suppressed cytochrome P450 2E1 (CYP2E1), hypoxia inducible factor α (HIF-1α) and inducible nitric oxide synthase (iNOS) which contributed to oxidative stress and restored sirtuin 1 (SIRT1)/AMP-activated protein kinase (AMPK)/peroxisome proliferator-activated receptor-gamma Co-activator-1α (PGC-1α) pathway-associated fatty acid oxidation in chronic ethanol-fed mice, which in turn ameliorated lipid peroxidation and macrosteatosis in liver. Take together these findings, DMB could serve as a novel and potential therapy for ALD in human beings.
INTRODUCTION

Alcoholism is a major cause of morbidity and mortality worldwide. In 2004 the ethanol consumption-associated chronic liver disease accounted for 3.8% of all deaths around the world (Rehm et al., 2009). The spectrum of ALD ranges from simple steatosis to severe alcoholic hepatitis, fibrosis, cirrhosis and ultimate hepatocellular carcinoma (Lieber, 2004). Accumulating evidence indicated that oxidative stress plays a critical role in ethanol-induced liver injury (Hoek and Pastorino, 2002). Metabolizing ethanol in liver is always accompanied with abundant ROS production, which initiates lipid peroxidation, glutathione depletion, abnormal methanionine metabolism and malnutrition. Cytochrome P450-2E1 (CYP2E1) and mitochondrial respiratory chain (MRC) have been considered as two major contributors of ROS production associating with ethanol-dependent oxidative stress (Lu and Cederbaum, 2008; Hoek, 2002). Usually CYP2E1 has been recognized to produce abundant ROS such as hydrogen peroxide and superoxide anion radical in the membrane of the endoplasmic reticulum (ER). However, in recent time increasing attention has been paid to the CYP2E1 within mitochondria, as it has been well documented that mitochondrial CYP2E1 augments local and cellular oxidative stress and acts as a critical pathogenic role in alcoholic liver diseases (Knockaert et al., 2011; Knockaert et al., 2011; Bansal et al., 2010). Increased mitochondria-located CYP2E1 causes the ROS overproduction, GSH depletion and dysfunction in mitochondria. Therefore, we can regard the specific CYP2E1 within mitochondria as a novel and potential target for preventing ALD. Mitochondria-mediated ethanol metabolism acts as another important role in oxidative stress (Hoek et al., 2002). Increased supply of mitochondrial NAD (P) H and reducing pressuring resulted from acute and chronic alcohol treatments significantly promote mitochondrial ROS formation in liver cells (Kurose et al., 1997;
Higuchi et al., 2001; Bailey et al., 2001). In addition, CYP2E1-induced increase in the proportion of O₂ uptake for oxidizing ethanol competes with mitochondrial electron transport in the utilization of O₂ (Ingelman-Sundberg et al., 1994), which consequently develop localized and transient hypoxia in the tissue, particularly in the pericentral region of the liver acinus (Arteel et al., 1997; Cunningham and Ivester, 1994). Such conditions of hypoxia and reoxygenation further strengthen ROS formation through mitochondrial respiratory chain. On the other hand, ethanol medicated activation of Kupffer cells generates reactive nitrosative stress (RNS) through induction of iNOS, which exacerbates the oxidative stress and mitochondrial dysfunction in liver (Venkatraman et al., 2004; McKim et al., 2003). Therefore, these findings suggested mitochondria as an important target of therapies for ALD. Currently, Chacko et al have treated ALD in mice with mitochondria-targeted antioxidant MitoQ, an artificial conjugate of ubiquinone with triphenylphosphonium cation (TPP⁺) which targets the ubiquinone to mitochondria (McKim et al., 2011). However, several researches indicated the cellular toxicity associated with TPP⁺.

DMB introduced in this study is a novel cationic antioxidant which comes from Chinese herb Cortex Phellodendri chinensis (CPC) that has a long history of traditional Chinese medicine use. Theoretically, DMB could be guided into mitochondrion by the high negative potential inside mitochondrion. Recent studies revealed that CPC has many pharmacological activities such as anti-microbial, anti-inflammatory, anti-diarrhoea and anti-cancer (Chia Ying et al., 2006; Shuanglai et al., 2011), but the particular biological properties of DMB are still elusive. Our study proposes DMB as a potential agent for treating ALD due to its special structure which contains both cationic and catechol groups (Fig. 1A). Series of in vitro and in vivo experiments were conducted to evaluate the biological activity of DMB in this work.
MATERIALS AND METHODS

Materials

Demethyleneberberine hydrochloride was synthesized in our laboratory slightly modified from Pan JF, et (Pan et al., 2001). The purity of DMB was more than 98% analyzed by HPLC (Parameters not shown). The structure was identified by MS, $^1$H-NMR and other methods (Supplemental Fig. 1). Total antioxidant capacity (T-AOC), alanine aminotransferase (ALT), thiobarbituric acid reactive substances (TBARS) and glutathione (GSH) test kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Rhodamine123 and DCFH-DA were products of Sigma Chemical Co. (St Louis, MO, USA). Other chemicals were of analytical grade.

Total antioxidant capacity (T-AOC) test

Antioxidant reduces Fe$^{3+}$ to Fe$^{2+}$, which coordinates with phenanthroline to form a stable and colored complex reaching the maximum absorbance at 520 nm. To determine the antioxidant property of DMB, T-AOC test was conducted according to the protocol of commercial kit.

Determining the mitochondria-targeted potential of DMB in vitro and in vivo with HPLC

HepG 2 cells (ATCC, USA) were maintained in Dulbecco’s modified Eagle’s medium (Gibco, USA) in a humidified incubator, supplemented with 10% fetal bovine serum and penicillin (100 U/ml) / streptomycin (100 mg/ml). When cells reached 80% confluence in the flasks, DMB was added with the final concentration of 50 μM for 30 min. Then washed cells with PBS buffer for three times and collected cells followed by isolating mitochondria and cytoplasm with the method of differential centrifugation (Frezza et al., 2007). The isolated mitochondria were washed with isolating solvent for 3 times to avoid the cytoplasmic contamination. DMB distributed in
mitochondria and cytoplasm was identified by HPLC (parameters not shown). Adult male ICR mice (8 weeks, 24-26 g) from College of Veterinary Medicine, Yangzhou University (Yangzhou, China) were housed in temperature- and humidity-controlled rooms. The mice were maintained at a 12-hour light/dark cycle and had free access to rodent chow and tap water. All procedures were approved by the Institutional Animal Care and Use Committee at China Pharmaceutical University and adhered to the Jiangsu Provincial Guidelines for the use of experimental animals. Mice were administrated with DMB (10 mg/kg) intravenously for once, and every individual liver were collected after 15min. The mitochondria and cytoplasm from livers were isolated by differential centrifugation as described above. DMB distributed in mitochondria and cytoplasm was identified by HPLC (parameters not shown).

**ROS determination**

HepG 2 cells were cultured in six-well plates under the same condition described above. With suitable confluence, cells were incubated with 50 μM DMB for 2h, after washed with PBS buffer for three times, H₂O₂ was then used to stimulate cells for another 15 minutes at the final concentration of 1mM. Cells were incubated with 5 μM DCFH-DA at 37°C for 30 min in the dark and then washed with PBS three times to remove excess dye (Yeligar et al., 2012; Taiji et al., 1997). Fluorescence Microscope and Flow Cytometry (FCM) were used to image and quantify the fluorescence intensity to reflect the ROS production.

**Mitochondrial membrane potential test with cell culture**

Mitochondrial membrane potential (MMP) was evaluated in HepG 2 cells cultured with medium containing 0.5% ethanol in the presence or absence of 50 μM DMB for 1 week. As an indicator of MMP, Rhodamine123 was used to evaluate the protective effect of DMB on ethanol-induced...
MMP decrease (Yan et al., 2007) by the detection of the fluorescence intensity with FCM.

**Acute alcoholic liver injury model**

The acute alcohol exposure model has been proposed as a predictive/screening tool for therapies against liver damage due to chronic alcohol intake. Adult male ICR mice (8 weeks, 24-26g) were divided into three groups-(1) Control, (2) Ethanol, (3) E+DMB (40 mg/kg, IP), n=8. The dosages were based on preliminary range-finding studies. All animals were exposed to ethanol (6 g/kg, IG) for three times at 12-h intervals, while mice in control group received saline as a vehicle control (Richard et al., 2014). Mice were treated with DMB 1h after each ethanol exposure, while other groups received an equal volume of vehicle.

**Chronic alcoholic liver injury model**

Adult male ICR mice (8 weeks, 24-26g) were divided into 3 groups, (1) Control, (2) LD Diet, (3) LD+DMB (40 mg/kg/day, IP), n=10, which were pair-fed isocaloric Lieber-DeCarli liquid diet (LD Diet) containing 0% or 36% ethanol by caloric content for 5 weeks to produce chronic alcoholic liver disease (CALD) (Nanji et al., 1989). The dosage of DMB was based on the preliminary range-finding studies. At sacrifice, animals were anesthetized with sodium pento-barbital (80 mg/kg, IP) 6 hours after ethanol administration. Blood was collected with cardiac puncture just prior to sacrifice. Liver tissues were harvested at the time of sacrifice.

**Serum ALT assay**

Serum was separated by centrifugation of 800g at 4°C for 10min after 40min standing at room temperature. Serum ALT activity was measured using commercially diagnostic kits.

**Histopathological analysis**

After sacrificed, small pieces of individual liver from same location were harvested, washed with
ice-cold saline and fixed in 4% neutral buffered formalin solution. Sections of 5 mm thickness were cut, deparaffinized, hydrated and stained with hematoxylin and eosin.

**Western blot analysis**

Mice liver tissue or isolated mitochondria were homogenized and sonicated in ice-cold lysis buffer (RIPA) containing protease inhibitor cocktail (Roche, Switzerland). Lysates were centrifuged at 10,000 g, 4°C and supernatant was collected, of which protein concentration was determined via the bicinchoninic acid (BCA) method. Equal amounts of protein (100 μg) were loaded on 10% polyacrylamide gel (29:1 acrylamide-bisacrylamide), separated by SDS-PAGE and transferred to PVDF membranes (Millipore, USA). The membrane was blocked for 1 h at room temperature in TBST buffer containing 5% BSA, and then incubated with the primary antibody in TBST with 5% BSA overnight at 4°C. Primary antibody at appropriate dilution ratio was used to detect CYP2E1 (Anbobio, USA), iNOS, HO-1, GAPDH (Bioworld, USA). With 3 times washing of TBST, membrane was incubated with the secondary antibody (Bioworld, USA) in TBST with 5% BSA, and exposed with ECL (Millipore, USA) after another 3 time washing of TBST.

**Immunohistochemistry**

Liver sections (10 μm) were mounted on glass slides. Sections were deparaffinized, and incubated in 3% H2O2 for 10 min to quench endogenous peroxidase activity. After blocking with normal goat serum for 20 min, the sections were stained with polyclonal rabbit antibody raised against CYP2E1 (1:100, Bioworld, USA) and HIF1α (1:20, BBI, Canada) at 4°C overnight respectively, followed by washed with PBS and incubated with goat antirabbit antibody at 37°C for 30 min. The antibody binding sites were visualized by incubation with DAB at room temperature for 10 min.
Electron microscopy

Liver tissue (1 mm$^3$) from the same location of the liver was removed and pre-fixed for 2 h at 4$\degree$C in 4% paraformaldehyde solution. The tissue was then rinsed in the same buffer and post-fixed in 1% osmium tetroxide. Post-fixation was followed by dehydration in ethanol, embedding in Epon 812 and polymerization. The tissue was cut using an ultramicrotome, stained with 1% uranyl acetate and lead citrate, and examined under a transmission electron microscope.

Evaluation of mitochondrial chemistry

Mitochondria were isolated by differential centrifugation of the liver homogenate (Frezza et al., 2007). The total protein of isolated mitochondria was measured by BCA method. Mitochondrial peroxidative product (TBARS), antioxidant defense (GPx/GSH), mitochondrial AST (mAST) activity were determined by the commercial kits according to the manufacturer’s instructions.

Mitochondrial swelling test

The assays were performed in 1 ml of the reaction media (250 mM sucrose, 3 mM sodium succinate, 5 mM KH$_2$PO$_4$, 0.3 mM CaCl$_2$) (Galindo et al., 2003). Changes of absorbance at 520 nm in 10 minutes were recorded after 0.5 mg of protein was added to the solution. Mitochondrial osmotic volume changes were presented by the decrease of absorbance at 520 nm. Damaged mitochondria reflected much less sensitivity to the Ca$^{2+}$ loading, which was demonstrated by blunted decrease of 520 nm absorbance.

Statistical analysis

All analyses were performed with Statistical Package for Social Sciences version 16.0 (SPSS, Chicago, IL). Data were expressed as mean ± SD. Statistical significance was determined by one-way ANOVA. In all statistical comparisons, a $P$ value <0.05 was used to indicate a
RESULTS

DMB was determined to be a mitochondria-targeted antioxidant.

In the first place, the antioxidant capability of DMB was detected by T-AOC and ROS tests. As shown in the result of T-AOC test, DMB exhibited antioxidant capacity in a concentration-dependent manner (Fig. 1B). In the ROS experiment the probe DCFH-DA only detects the intracellular ROS, reflecting the antioxidant activity of DMB in cells. As a result, DMB neutralized H$_2$O$_2$-originated ROS by 55% in HepG 2 cells as compare to group treated with H$_2$O$_2$ alone (Fig. 1C).

In the second place, given the cationic structure of DMB, we performed HPLC experiment to address whether DMB could target to mitochondria. As a result, DMB was detectable significantly in both mitochondria and cytoplasm isolated from HepG 2 cells (Fig. 1D). Due to that the volume of mitochondria is smaller than that of cytoplasm, the concentration of DMB in mitochondria is suggested to be much higher than that in cytoplasm. Then we evaluated the effect of DMB on mitochondria by testing MMP in HepG 2 cells exposed to 0.5% ethanol for 1 week. Control cells displayed strong Rhodamine123 fluorescence intensity, reflective of viable intact cells (Fig. 1E), while a small amount of cells located in the low-Rhodamine123-fluorescence (M1) population, reflective of damaged cells (Fig. 1E). Ethanol stimulation caused fluorescence migration to the M1 population (Fig. 1E), while, inspiringly, co-incubation in the presence of 50 μM DMB significantly reversed such migration, protecting HepG2 cells from the decline in MMP produced by ethanol (Fig. 1E).

DMB protected liver mitochondria from oxidative stress in acute ethanol-treated mice.
Based on the above findings, the biological activity of DMB in vivo was investigated with binge drinking mice. As shown by H&E staining, binge ethanol caused microsteatosis, swelling and even apoptosis in liver cells. DMB treatment (40 mg/kg) remarkably attenuated these histopathological damages (Fig. 2A). Elevated serum ALT by binge drinking was also blunted in mice after DMB treatment (Fig. 2B).

Mitochondria have long been recognized as the major targets of ethanol-induced oxidative stress (Hoek et al., 2002). Our results showed that binge drinking significantly reduced mitochondrial GSH and Gpx activity by 33%, and further elevated TBARS formation in mitochondria by 250% (Fig. 2C). DMB treatment completely blocked the decline of GSH, Gpx activity and the TBARS formation in mitochondria (Fig. 2C). Blunted decrease in 520 nm absorbance indicated the swelling of mitochondria, suggesting that mitochondrial membrane was disturbed by ethanol-induced ROS. DMB treatment alleviated ethanol-mediated mitochondrial swelling, monitored by increased rate of the decline in 520 nm absorbance (△520 nm) (Fig. 2D-a). In addition, binge ethanol treatment in mice decreased mitochondrial AST (mAST) activity by 60%, which suggested that ethanol ruptured mitochondrial membrane resulting in the mAST leaking out (Fig. 2D-b). DMB treatment significantly recovered mAST activity by 50% (Fig. 2D-b).

Electron microscopy images showed that the DMB treatment profoundly ameliorated ultrastructural damages of mitochondria. Under the condition of binge ethanol, the membrane and cristae of mitochondria were ruptured and several mitochondria were even transformed to be megamitochondrion (Fig. 2E). Moreover, endoplasmic reticulum became swollen and thin, and lots of lipid droplets were observed in binge ethanol-treated group (Fig. 2E). All these abnormal ultrastructural changes were significantly improved by DMB treatment. Rich endoplasmic
reticulum, normal mitochondria but few lipid droplets were found in hepatocytes from DMB-treated mice (Fig. 2E). Therefore, the results supported that DMB could prevent alcoholic liver injury \textit{in vivo}, at least partially, through protecting mitochondria from ethanol-induced oxidative stress.

**DMB alleviated chronic alcoholic liver injury.**

Chronic alcoholic liver injury model was set up to mimic the initial pathogenesis of ALD in human beings due to long-term drinking (Lieber and DeCarli, 1989; Brandon-Warner et al., 2012). Mice were pair-fed with either ethanol or control liquid diets for 5 weeks in the presence or absence of DMB (40 mg/kg/day, IP). The liver from chronic ethanol-treated group looks rough, swollen and xanthic (Fig. 3A). The ratio of liver to body weight and serum ALT activity were increased about 1.2 and 2.3-fold respectively by chronic ethanol exposure as compared to pair-fed controls, both of which were attenuated by DMB treatment (Fig. 3B, C). In accordance, H&E staining revealed that chronic drinking in mice caused significant micro/macro-steatosis and hepatocellular ballooning (Fig. 3D). The severe pathological changes were substantially alleviated by DMB treatment, which revealed a protective effect of DMB on chronic alcoholic liver disease (CALD) (Fig. 3D).

**DMB suppressed CYP2E1, HIF-1α and iNOS which contributed to ethanol-dependent oxidative stress.**

That DMB treatment (40 mg/kg/day) ameliorated oxidative stress in response to chronic alcohol consumption was reflected by decreasing hepatic TBARS formation by 25% in chronic ethanol feeding mice (Fig. 4A). Induction of CYP2E1 have been well documented to be a central pathway that contributes to ethanol-mediated oxidative stress (Lieber, 2004; Hoek and Pastorino, 2002; Lu...
and Cederbaum, 2008). Elevated CYP2E1 by ethanol generates excessive reactive oxygen species such as the hydrogen peroxide and superoxide anion radical. Our results showed that DMB treatment dramatically reduced the total CYP2E1 protein expression (Fig. 4B-a) and blocked the distribution of CYP2E1 around the veins (Fig. 4B-b). In the past decades, CYP2E1 has been recognized to generate ROS in the membrane of the endoplasmic reticulum (ER) for a long time, but currently, increasing attention has been paid to CYP2E1 within mitochondria. Many studies reported that mitochondrial CYP2E1 augmented local and cellular oxidative stress and acted as a critical pathogenic role in alcoholic liver diseases (Knockaert et al., 2011; Knockaert et al., 2011; Bansal et al., 2010). A recent study even indicated that mitochondrial CYP2E1 made more contribution to the pathogenesis of ALD than microsomal CYP2E1 did (Knockaert et al., 2011).

We investigated the isoforms of CYP2E1 located in mitochondria (mtCYP2E1), phosphorylated CYP2E1 (P-mt CYP2E1) and truncated mtCYP2E1 (T-mt-CYP2E1). Western blot showed that both forms of mtCYP2E1 were reduced by DMB treatment in mice fed with chronic ethanol (Fig. 4C). Consequently, CYP2E1-associated hypoxia was alleviated by DMB, which was reflected by reduced HIF-1α induction around the veins (Fig. 4D). In addition, iNOS induced by chronic alcohol consumption in mice was also suppressed after DMB treatment (Fig. 4E).

**DMB prevented alcoholic fatty liver by restoring SIRT1/AMPK/PGC1-α pathway-associated fatty acid oxidation.**

The onset and progression of ALD is usually characterized by steatosis (Chia Ying et al., 2006). In accordance, the TG content in liver from ethanol-treated mice were significantly increased 200% compared to control, while DMB treatment reduced the TG by about 50% (Fig. 5A). Chronic alcohol consumption resulted in extensive accumulation of Oil Red O stained lipids around the
central vein and periportal region, which was blunted by DMB treatment apparently (Fig. 5B). In the liver, AMPK could increase fatty acid oxidation via the activation of PGC-1α which coactivates the expression of enzymes involved in fatty acid oxidation such as CPT1a, ACO, MCAD (Joanne et al., 2008). Recent studies demonstrated that SIRT1 acted upstream of AMPK and also could directly activate PGC-1α to promote fatty acid utilization (Banks et al., 2008; Lan et al., 2008; Amat et al., 2009). Our study revealed that DMB substantially reversed the inhibitory effects of chronic ethanol feeding on hepatic SIRT1, p-AMPK and PGC-1α expression at protein or mRNA level in mice (Fig. 5C-a, b). We further determined whether the activated SIRT1/AMPK/PGC1-α pathway resulted in altered expression of genes associated with fatty acid oxidation by real-time PCR. Results showed that the carnitine palmitoyltransferase 1a (CPT1a), acyl-CoA oxidase (ACO) and mitochondrial medium-chain acyl-CoA dehydrogenase (MCAD) mRNAs expression of DMB-treated group were 2-fold, 9-fold and 2-fold higher than those of the model group, respectively (Fig. 5D).
DISCUSSION

The present study characterized the diverse hepato-protective effects of DMB on ALD. We identified a novel and unique mechanism involving the attenuation of hepatic oxidative stress and steatosis partially through the mitochondria-targeted antioxidation, down-regulation of CYP2E1 and activation of SIRT1/AMPK/PGC-1α pathway-associated fatty acid oxidation. Such action differs from that of the well-known anti-oxidants S-adenosyl methionine and tocopherol, which restores glutathione content (Tilg and Day, 2007; Altavilla et al., 2005).

Alcohol-induced liver injury enhances the susceptibility of the liver to develop steatohepatitis, fibrosis, cirrhosis and carcinoma (Lieber, 2004). Since mitochondria are both sources and targets for reactive oxygen and nitrogen species (ROS/RNS), it is reasonable to conclude that they play a central role in the pathophysiology of ethanol-mediated hepatotoxicity (Hoek et al., 2002; Lieber, 2000]. Recent studies have closely linked the production of ROS to mitochondrial DNA, membrane and protein damage and alcohol-dependent metabolic derangements in the liver. On the basis of these findings we thought a mitochondria-targeting antioxidant could potentially attenuate pathological changes in response to alcoholism. DMB introduced in this study has long been identified as the ingredient of Cortex Phellodendri chinensis as well as a major metabolite of berberine, another traditional Chinese medicine, in vivo (Feng et al., 2006). However, this is for the first time that DMB was recognized as a natural mitochondria-targeted antioxidant for treating ALD in our study. Firstly, the mitochondria-targeting potential and antioxidant activity of DMB were demonstrated by a series of in vitro experiments. Secondly, we further confirmed that DMB potentially targeted to liver mitochondria in vivo and significantly protected liver mitochondria...
from acute ethanol-induced oxidative damages in mice. Thirdly, DMB was determined to treat chronic alcoholic liver injury that resembled the initial pathogenesis of ALD in human beings due to long-term drinking, which suggested that DMB could serve as a potential therapy for human ALD.

The detrimental consequences of drinking alcohol on liver, especially of heavy and/or long-term consumption, have been well established. Heavy and chronic ethanol consumption caused ALD in approximately 20% of individuals. Ethanol-induced robust ROS are predominantly produced by CYP2E1 and mitochondrial respiratory chain in liver (Hoek et al., 2002). Based on the etiopathogenesis of alcoholic liver injury, it is reasonable to regard DMB as an effective therapy for ALD which combats mitochondrial ROS formation as well as CYP2E1-dependent oxidative stress. An interesting result in this study was that DMB treatment significantly reduced two isoforms of mitochondrial CYP2E1- a highly phosphorylated 52-kDa form and a shortened 40-kDa NH2-terminal truncated form (Fig. 4B-a, C) (Knockaert et al., 2011). Recent studies indicated that mitochondrial CYP2E1 could be induced by ethanol exposure and contributed to the pathogenesis of ALD and the localization of CYP2E1 within mitochondria resulted in mitochondrial ROS overproduction, GSH depletion and membrane potential decrease (Knockaert et al., 2011; Knockaert et al., 2011; Bansal et al., 2010). cAMP-dependent phosphorylation of CYP2E1 by protein kinase A (PKA) has been thought to result in increasing the association of the protein with cytoplasmic Hsp70 and Hsp90 chaperones and favoring its binding to mitochondrial translocases outer membrane transporters (TOM) (Robin et al., 2002; Anandatheerthavarada et al., 2009). Although DMB can target to mitochondria in hepatocytes as shown in the HPLC charts, amounts of DMB still reside in cytoplasm. We propose that DMB might decrease mitochondrial
CYP2E1 by interfering the phosphorylation of CYP2E1 induced by PKA in the cytoplasm. On the other hand, it is also possible that the DMB inside mitochondria disturbed the mitochondria-location and further truncation of CYP2E1, which needs further investigation.

Accumulation of lipids as micro- and macrovesicles and the distinctive localization of lipid vesicles determined the characteristic tissue pathology resulted from chronic alcohol consumption.

DMB-mediated inhibition of both micro- and macrosteatosis suggested the direct or indirect interference with the pathways related to lipid metabolism in the liver. Both SIRT1 and AMPK have been implicated in the development of alcoholic fatty liver (Garci’a-Villafranca et al., 2008; Lieber et al., 2008; You et al., 2004). The present study indicated that DMB exerts its protective action against ethanol-induced liver steatosis by turning on the hepatic SIRT/AMPK signaling system (Fig. 5C). SIRT1 and AMPK actions converge through PGC-1α to enhance fatty acid oxidation, suggesting that SIRT1/AMPK/PGC-1α stimulating may serve as a key mechanism for alleviating alcoholic fatty liver. Consequently, the mRNA levels of several genes associated fatty acid oxidation and lipid transport were restored by DMB (Fig. 5D). These data indicated that DMB ameliorated hepatosteatosis in response to chronic alcohol consumption by activating SIRT1/AMPK/PGC-1α pathway-associated fatty acid oxidation. However, the specific mechanism by which DMB regulates this pathway needs to be confirmed by more evidence. Interestingly, DMB has also been identified as a major metabolite of berberine (BBR) in vivo (Feng et al., 2006).

BBR is a well recognized AMPK agonist to attenuate lipid accumulation in liver (Yang et al., 2013; Turner et al., 2008). Even though BBR has studied for many years, it remains unclear how BBR activates AMPK. But we believe that DMB shares the structure-activity relationship with BBR in part.
In summary, this study identified a natural cationic antioxidant DMB as a potential agent to treat ALD in mice and uncovered novel mechanisms of DMB on ameliorating ethanol-dependent oxidative stress which was distinct from other antioxidants. These findings raise the possibility that DMB might be beneficial in liver disease associated with increased oxidative stress, including alcoholic/nonalcoholic steatohepatitis and chronic hepatitis C. Further studies are needed to confirm the effects of DMB against these diseases.
AUTHOR CONTRIBUTION

Participated in research design: Pengcheng Zhang, Ruiyan Li, Xie Liu, Yubin Zhang

Conducted experiments: Pengcheng Zhang, Xiaoyan Qiang, Miao Zhang, Dongshen Ma, Zheng Zhao, Cuisong Zhou

Performed data analysis: Pengcheng Zhang, Huan Chen

Wrote or contributed to the writing of the manuscript: Pengcheng Zhang, Yubin Zhang
REFERENCES


FOOTNOTES

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FIGURE LEGENDS

Fig. 1 DMB was determined to be mitochondria-targeted antioxidant.

(A) Structure of DMB. (B) T-AOC of DMB. (C) Representative images of ROS fluorescent staining with DCFH-DA (original magnification, 200×). Flow cytometric histograms are shown in the right panel. (D) HPLC analysis of DMB in mitochondria isolated from HepG 2 cells. (E) Flow cytometric histograms of MMP indicated by Rho123. M1: low Rhodamine123 fluorescence intensity. Bar graph represents the quantified fluorescence intensity. Values represent means ± SD (n=3). ##P < 0.001 versus control; ###P < 0.001 versus H2O2 (1mM) or EtOH group.

Fig. 2 DMB protects liver mitochondria from binge ethanol-induced oxidative damage in mice.

(A) Liver H&E staining (original magnification, 400×). Arrow ①, microsteatosis; arrow ②, apoptosis. (B) Serum alanine aminotransferase (ALT) levels. (C) Mitochondrial levels of GSH, GPx activity and TBARS. (D-a) Mitochondrial swelling; (D-b) Mitochondrial AST activity. (E) Mitochondrial electron microscope (EM ×1700). M: mitochondria, N: nucleus, ER: endoplasmic reticulum, LD: lipid droplet. Values represent means ± SD (n=3-8). ##P < 0.01, ###P < 0.001 versus control. *P < 0.05; **P < 0.01, ***P < 0.001 versus ethanol.

Fig. 3 DMB prevents chronic alcohol consumption-induced liver injury in mice.

(A) Liver tissues from each group. (B) Ratios of liver to body weight. (C) Serum ALT activity. (D) Liver H&E staining (original magnification, 400×). Arrow ①, macrosteatosis; arrow ②, inflammation.

Fig. 4 DMB suppresses CYP2E1, HIF-1α and iNOS which contribute to ethanol-mediated oxidative stress.
(A) Hepatic levels of TBARS. (B-a) Western blot analysis of total CYP2E1 in liver. GAPDH was used as loading control; (B-b) IHC analysis of CYP2E1 location in liver (400 ×). (C) Mitochondrial CYP2E1 was distinguished by western blot and VDAC was used as loading control for mitochondrial protein. β-actin is an indicator of cytoplasmic protein. (D) IHC analysis of HIF-1α location in liver (400 ×). (E) Western blot analysis of iNOS in liver. Values represent means ± SD (n=6-8). *p < 0.05, **p < 0.01 versus control. *p<0.05, **p<0.01 versus LD Diet.

Fig. 5 DMB attenuates alcoholic fatty liver and restores SIRT1-AMPK-PGC1-α pathway.

(A) Hepatic TG level. (B) Oil Red O liver staining (Original magnification, 200× and 400×). (C) Western blots analysis of SIRT1, p-AMPK/AMPK, and PGC-1α proteins. GAPDH was used as loading control. (D) Relative mRNA levels of CPT1a, ACO and MCAD. Cyclophinlin was used as endogenous reference. Values represent means ± SD (n=4-8). *p<0.05, **p<0.01 versus control. *p<0.05, **p<0.01 versus LD Diet.
Table 1. Primer sequences for PCR analysis

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<tr>
<td>ACO</td>
<td>5’-TCCAGACTTCCAACATGAGGA-3’</td>
<td>5’-CTGGGCGTAGGCTGCAATTA-3’</td>
</tr>
<tr>
<td>MCAD</td>
<td>5’-TCGAAAGCGTCACAAGCAG-3’</td>
<td>5’-CACCACAACCTTTCCCGAATGT-3’</td>
</tr>
<tr>
<td>SIRT1</td>
<td>5’-GGGTTCCTGTCTCCTGTG-3’</td>
<td>5’-GAATGGTTCTTGAGTCTTT-3’</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>5’-TGCCATTGTTAAGCCGAG-3’</td>
<td>5’-TTGGGTTGCTTGTGGTGAC-3’</td>
</tr>
<tr>
<td>Cyclophilin</td>
<td>5’-CCATCGTGCATCAAGGACTTCAT-3’</td>
<td>5’-CTTGCCCATCCAGCCAGGTCTT-3’</td>
</tr>
</tbody>
</table>
Figure 1
Figure 2
Figure 3
**Figure 4**

(A) Graph showing the levels of sHRS (nmol/g protein) across different conditions. The bars represent the control, LD Diet, and LD+DMB groups. The error bars indicate variability.

(B) Western blot analysis of Total CYP2E1. The bands are observed at 54 KD and 36 KD.

(C) Western blot analysis of P-mtCYP2E1 and T-mtCYP2E1. The bands are observed at 40 KD, 42 KD, and 30 KD. The graphs show the relative levels of P-mtCYP2E1 and T-mtCYP2E1 in different conditions.

(D) Immunohistochemical staining of HIF1α in different conditions. The images are magnified to 400x.

(E) Western blot analysis of iNOS. The bands are observed at 130 KD and 36 KD. The graphs show the relative levels of iNOS in different conditions.
Figure 5

A

B

Control

LD Diet

LD+DMB

200x

C

D

110 KD

63 KD

97 KD

36 KD

SIRT 1

p-AMPK

AMPK

PGC1α

GAPDH

Relative levels of SIRT1, pAMPK, AMPK, and PGC1α

Relative mRNA level

Control

LD Diet

LD+DMB

Control

LD Diet

LD+DMB

CPT1α

ACO

MCAD