Antcin H Protects Against Acute Liver Injury
Through Disruption of the Interaction of c-Jun-N-Terminal Kinase with Mitochondria

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Abstract

Aim: Antrodia Camphorate (AC) is a mushroom that is widely used in Asian countries to prevent and treat various diseases, including liver diseases. However, the active ingredients that contribute to the biological functions remain elusive. The purpose of the present study is to test the hepatoprotective effect of Antcin H, a major triterpenoid chemical isolated from AC, in murine models of acute liver injury.

Results: We found that Antcin H pretreatment protected against liver injury in both acetaminophen (APAP) and galactosamine/tumor necrosis factor (TNF)α models. More importantly, Antcin H also offered a significant protection against acetaminophen-induced liver injury when it was given 1 h after acetaminophen. The protection was verified in primary mouse hepatocytes. Antcin H prevented sustained c-Jun-N-terminal kinase (JNK) activation in both models. We excluded an effect of Antcin H on acetaminophen metabolism and TNF receptor signaling and excluded a direct effect as a free radical scavenger or JNK inhibitor. Since the sustained JNK activation through its interaction with mitochondrial Sab, leading to increased mitochondrial reactive oxygen species (ROS), is pivotal in both models, we examined the effect of Antcin H on p-JNK binding to mitochondria and impairment of mitochondrial respiration. Antcin H inhibited the direct effect of p-JNK on isolated mitochondrial function and binding to isolated mitochondria.

Innovation and Conclusion: Our study has identified Antcin H as a novel active ingredient that contributes to the hepatoprotective effect of AC, and Antcin H protects against liver injury through disruption of the binding of p-JNK to Sab, which interferes with the ROS-dependent self-sustaining activation of MAPK cascade. Antioxid. Redox Signal. 26, 207–220.

Keywords: Antrodia Camphorate, Antcin H, liver injury, c-Jun-N-terminal kinase, mitochondria, ROS

Introduction

Acetaminophen (APAP) is one of the most commonly used analgesic drugs. APAP hepatotoxicity in humans was first reported in 1960s (7). Since then, APAP has become the leading cause of acute liver failure in many developed countries, including the United States and most of the European countries (11, 22, 23). N-acetyl-p-benzoquinone (NAPQI), an electrophilic reactive metabolite of APAP produced mainly by CYP2E1 metabolism, plays a pivotal role in activation of downstream toxic signaling pathways, including glutathione (GSH) depletion, NAPQI-protein adduct formation (6, 13), reactive oxygen species (ROS) accumulation, sustained c-Jun-N-terminal kinase (JNK) activation and its mitochondria translocation (12, 15), and increased mitochondrial membrane permeability transition, which eventually leads to necrotic cell death (43–45).

Tumor necrosis factor (TNF)α, a proinflammatory cytokine, is suggested to play a critical role in hepatocellular death in many conditions, such as immune-mediated and alcoholic and nonalcoholic fatty liver diseases (3, 36, 38). Galactosamine (GalN), as a hepatocyte-specific sensitizer,
Antrodia Camphorate (AC) is a mushroom which is widely used in Asian countries to prevent and treat various diseases, including liver diseases. However, the active ingredients that contribute to the biological functions of AC remain elusive. We found for the first time that Antcin H is highly effective against either acetaminophen (APAP) or galactosamine (GalN)/tumor necrosis factor (TNFα)-mediated hepatotoxicity. Our study has identified Antcin H as a novel active ingredient that contributes to the hepatoprotective effect of AC, and Antcin H protects against liver injury through the disruption of the binding of p-JNK to Sab, which consequently interferes with the mitochondrial reactive oxygen species-dependent self-sustaining activation of MAPK cascade.

Enhances TNFα-mediated hepatotoxicity. GalN/TNFα-induced liver injury is a useful model for investigating innate immune-mediated apoptotic hepatotoxicity. In the GalN/TNFα model, sustained JNK activation has also been identified as a key event to trigger mitochondrial pathway-dependent apoptotic death of hepatocytes (14, 41, 43, 45). Targeting the key signaling pathways, such as JNK activation or its effects on mitochondria, may be an attractive approach to manage drug-induced and immune-mediated hepatotoxicity.

Folk medicines have long been used to manage various diseases, including liver disease. However, the scientific validation of their efficacy and the mechanistic interpretations are needed. Folk medicine is suggested to be a rich source for developing evidence-based chemopreventive or therapeutic agents. Antrodia Camphorate (AC) is a rare and precious medicinal mushroom found in Taiwan. As a folk medicine, AC is used widely in Asian countries for the treatment of various diseases, including liver disease (24). The chemistry of AC has been extensively investigated and it has been found that AC is rich in ergostane and lanostane triterpenoids (25, 31).

Antcin H is one of the main terpenoids isolated from AC. It has been shown that Antcin H possesses anti-inflammatory and insecticidal activities (5, 26, 35). We hypothesized that Antcin H could be a major active component of AC that may contribute to its hepatoprotective activity. In present study, the protective effect of Antcin H against drug-induced and immune-mediated hepatotoxicity has been evaluated using both APAP- and GalN/TNFα-induced acute liver injury in mouse models. The results demonstrate for the first time that Antcin H is highly effective against either APAP- or GalN/TNFα-mediated liver toxicity. Mechanistically, the hepatoprotective activity of Antcin H was attributed to its ability to directly disrupt the interaction between JNK and mitochondria and prevent JNK-mediated mitochondrial dysfunction.

**Results**

**Antcin H protects against APAP- or GalN/TNFα-induced hepatotoxicity in vivo and in vitro**

Our pilot study demonstrated that the ethanol extract of AC offered a significant protective effect on APAP-induced liver injury (data not shown). To identify the active compound that contributed to the hepatoprotective activity of AC, Antcin H, a major component of AC, was evaluated using both APAP- and GalN/TNFα-induced liver injury models.

For the APAP-induced liver injury model, we first investigated the protective effect of pretreatment with Antcin H. Antcin H was given by i.p. injection 1 h before APAP treatment. As shown in Figure 1A, 24 h after APAP treatment, the very high serum alanine aminotransferase (ALT) in vehicle controls was nearly abolished by pretreatment with 25 mg/kg or 50 mg/kg Antcin H. In line with the changes of ALT, the histological analysis showed that APAP-induced severe zone III necrosis of hepatic lobules was dramatically attenuated by the pretreatment with 25 or 50 mg/kg Antcin H (Fig. 1B).

Having established the preventive efficacy of Antcin H, we next explored the therapeutic potential of Antcin H against APAP-induced hepatotoxicity by administering APAP 1 h before Antcin H. Since maximal covalent binding and GSH depletion are seen by 1 h after APAP (27), a protective effect is unlikely to be due to inhibition of NAPQI production. As shown in Figure 1C and D, a significant protection was also observed in this therapeutic setting. To confirm that the effect of Antcin H was on hepatocytes, we assessed the effect of Antcin H 2 h after treatment of cultured mouse hepatocytes followed by 2 h of exposure to APAP and then changed to Antcin H containing media without APAP. Cell death at 24 h was decreased by Antcin H in a dose-dependent manner (Fig. 1E).

We next determined if Antcin H is also capable of countering the hepatotoxicity triggered by GalN/TNFα, which occurs by a different mode of cell death (apoptosis) and is independent of drug metabolism. Mice received Antcin H i.p. twice, 6 and 1 h, before GalN/TNFα administration. The liver damage was examined 6 h after GalN/TNFα exposure. Increased ALT level (Fig. 2A) and histological injury (Fig. 2B) in controls were significantly ameliorated or negligible in Antcin H pretreated mice. In the primary mouse hepatocyte culture model, cotreatment with Antcin H significantly inhibited apoptosis induced by actinomycin D/TNFα (Fig. 2C). Thus, these results demonstrate that Antcin H has protective effects against either APAP necrosis- or TNFα-mediated apoptotic hepatotoxicity in vivo and in vitro.

**Antcin H inhibits sustained JNK activation induced by APAP or GalN/TNFα**

To decipher the mechanisms of the hepatoprotective activity of Antcin H in the APAP model, we assessed the effect of Antcin H on APAP-induced GSH depletion and covalent binding. As shown in Figure 3A, vehicle-treated and Antcin H-treated mice were found to have a comparable level of GSH, whereas APAP treatment led to a significant reduction of GSH at 1 h as expected, but GSH depletion by APAP was not affected by Antcin H exposure. Furthermore, the formation of NAPQI-protein adducts by APAP was also not significantly affected by Antcin H (Fig. 3B). These results suggested that protection of Antcin H was not due to blocking the toxic metabolism of APAP.

Activation of JNK and its mitochondrial translocation are important in exacerbating APAP-induced hepatotoxicity (12, 15, 41, 43, 45). It is well established that APAP induces rapid mitochondrial impairment and increased O2•− production; released H2O2 then activates MAP3K leading to JNK activation. p-JNK then interacts with the mitochondrial outer
membrane protein, Sab, which induces further mitochondrial impairment and ROS production (29, 42, 43).

We therefore investigated whether APAP-induced JNK activation was attenuated by Antcin H. JNK activation was not seen in phosphate-buffered saline (PBS)-treated and Antcin H-treated control mice. As expected, APAP exposure caused a rapid JNK activation (as early as 15 min). Treatment with Antcin H did not significantly inhibit the early activation of JNK after APAP injection at 15 and 30 min (Fig. 3C), but nearly abolished activation of JNK after APAP injection at 1, 2, and 4 h (Fig. 3D), suggesting Antcin H might mainly target the sustained JNK activation rather than the initial or transient event of JNK activation induced by APAP. The latter is known to be a consequence of direct toxicity of APAP on mitochondria leading to ROS release and activation of MAPK (15).

In addition, the inhibitory effect of Antcin H on JNK activation was also found in the primary mouse hepatocyte culture model (Fig. 3F). We further evaluated mitochondrial JNK translocation using cytoplasmic and mitochondrial fractions from mice treated with APAP and/or Antcin H. Consistent with the above results, a dramatic reduction in p-JNK by Antcin H was observed in both the mitochondria and cytoplasm (Fig. 3E).

For GalN/TNFα-induced liver injury, we first assessed if Antcin H affected TNF receptor signaling. As shown in Figure 4A, TNFα alone induced a transient increase of p-IκBα and decrease of IκBα, which were not influenced by Antcin H treatment. In addition, the transient JNK phosphorylation after TNFα treatment was not suppressed by Antcin H. It is also well established that sustained JNK activation also plays a key role in GalN/TNFα-mediated toxicity (14, 41). In this case, the p-JNK interaction with Sab leads to mitochondrial ROS production and sustained JNK activation, which then modulates Bcl-2 family to induce mitochondria outer membrane permeabilization and apoptosis (20).

FIG. 1. Antcin H protects against liver injury induced by APAP. (A, B) Mice received 25 and 50 mg/kg Antcin H i.p. in corn oil 1 h before APAP (300 mg/kg), and serum ALT and representative histology (H&E) determined at 24 h. ***p < 0.001, n = 3–4 mice per group. (C, D) Antcin H (50 mg/kg) was administered 1 h after APAP (300 mg/kg), and serum ALT and histology determined at 24 h. **p < 0.01, n = 6 mice per group. (E) Cultured PMH were pretreated with 50 and 75 μM Antcin H for 2 h and then switched to APAP (10 mM) plus Antcin H. Necrotic cells (SYTOX Green positive) were counted in 10 different fields per dish at 24 h. *p < 0.05 (n = 3 experiments). ALT, alanine aminotransferase; APAP, acetaminophen; H&E, hematoxylin and eosin. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars
We therefore measured the effect of Antcin H on the sustained JNK activation. Liver samples were collected 30 min and 1 and 2 h after GalN/TNFα treatment, and the phosphorylation level of JNK was measured by Western blot. Phosphorylation of JNK in whole liver tissue homogenate and cytoplasmic and mitochondrial fractions (Fig. 4B, C) was substantially inhibited by Antcin H exposure. Thus, inhibition of JNK by Antcin H was found in both APAP- and GalN/TNFα-induced acute liver injury. Given the similar role of activated JNK on mitochondria in both models, the APAP model was used to further decipher the mechanisms of JNK inhibition by Antcin H in the subsequent experiments.

JNK is not the direct target of Antcin H

The above data showed that Antcin H markedly inhibited the sustained activation of JNK. We therefore assessed if Antcin H is a direct inhibitor of p-JNK activity. To test this hypothesis, we carried out an in vitro kinase assay using c-Jun fusion protein as a substrate of JNK and SP600125, a known p-JNK inhibitor, as a positive control. Cytoplasmic fraction containing p-JNK was isolated from APAP-treated (2 h) mice and then incubated with different concentrations of Antcin H (50, 75, and 100 μM) and Act D (0.5 μg/ml)/TNFα (5 ng/ml) simultaneously. After 6 h, percent apoptosis was determined (Hoechst 33248). *p<0.05, **p<0.01 (n=3 experiments). GalN, galactosamine; TNF, tumor necrosis factor. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

Antcin H protects against the liver injury induced by GalN/TNFα. (A, B) Mice were pretreated with Antcin H (25, 50 mg/kg in DMSO) twice, 6 and 1 h, before GalN (800 mg/kg)/TNFα (12 μg/kg) i.p. After 6 h, serum ALT level and liver histology (H&E) were determined ***p<0.001(n=3 mice per group). (C) Cultured PMH were treated with different concentrations of Antcin H (50, 75, and 100 μM) and Act D (0.5 μg/ml)/TNFα (5 ng/ml) simultaneously. After 6 h, percent apoptosis was determined (Hoechst 33248). *p<0.05, **p<0.01 (n=3 experiments). GalN, galactosamine; TNF, tumor necrosis factor. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

Antcin H blocks mitochondrial ROS generation induced by APAP, but is not a direct scavenger or antioxidant

JNK is intricately regulated by a network of kinases and phosphatases. We and others have previously shown that the interaction of p-JNK with Sab increases mitochondrial ROS (4, 42). We next measured ROS levels by using flow cytometry in primary mouse hepatocytes (PMH) after 1 h APAP treatment. Increased levels of cellular ROS were observed in APAP-treated PMH, while Antcin H significantly alleviated ROS accumulation to the basal level, although not directly addressing mitochondrial ROS (Fig. 6A).

However, when liver cytoplasm harvested 2 h after APAP (containing p-JNK) was incubated with MitoSOX-loaded normal liver mitochondria, the expected (42) increase in O2− was observed and was blocked by Antcin H (Fig. 6B). This effect could be due to Antcin H be a radical or ROS scavenger causing removal of ROS or it could be due to Antcin H interfering with the production of ROS by interfering with p-JNK interaction with Sab.

Therefore, we addressed whether Antcin H is a radical scavenger or directly scavengers ROS. Scavenging of DPPH radical, which is a stable free radical, has been used for assessing if a compound is a radical scavenger (46). We compared Antcin H with BHA, a well-known antioxidant, and we found no evidence that Antcin H is a radical scavenger compared to BHA, which was a potent inhibitor (Fig. 6C). To further verify that Antcin H does not directly inhibit intramitochondrial ROS production, we examined its effect on

FIG. 2. Antcin H protects against the liver injury induced by GalN/TNFα. (A, B) Mice were pretreated with Antcin H (25, 50 mg/kg in DMSO) twice, 6 and 1 h, before GalN (800 mg/kg)/TNFα (12 μg/kg) i.p. After 6 h, serum ALT level and liver histology (H&E) were determined ***p<0.001(n=3 mice per group). (C) Cultured PMH were treated with different concentrations of Antcin H (50, 75, and 100 μM) and Act D (0.5 μg/ml)/TNFα (5 ng/ml) simultaneously. After 6 h, percent apoptosis was determined (Hoechst 33248). *p<0.05, **p<0.01 (n=3 experiments). GalN, galactosamine; TNF, tumor necrosis factor. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars
FIG. 3. The effects of Antcin H on the metabolism of APAP and JNK activation. (A) Liver homogenate GSH 1 h after APAP injection was determined in control mice with or without Antcin H (50 mg/kg) pretreatment 1 h before APAP (300 mg/kg) i.p. **p < 0.01, n = 3. (B) Immunoblot of time course of NAPQI-protein adducts in liver extracts obtained 1 h after APAP (300 mg/kg) with or without pretreatment with Antcin H (50 mg/kg). Bar graph shows densitometry of total adducts/GAPDH of n = 3. (C) Initial p-JNK in samples with or without pretreatment with Antcin H. (D, E) Time course of p-JNK in samples from B, using whole liver homogenate (D) and cytoplasm and mitochondria (E). Bar graphs show densitometry of p-JNK/JNK or p-JNK/PHB1 of n = 3. (F) PMH were pretreated with Antcin H or DMSO vehicle for 2 h. Media then changed to APAP (10 mM) without Antcin H, and whole cell lysates were collected at 1, 2, and 4 h for Western blot. Bar graph shows densitometry of p-JNK/JNK of n = 3. ***p < 0.001. JNK, c-Jun-N-terminal kinase; GSH, glutathione; NAPQI, N-acetyl-p-benzoquinone; PMH, primary mouse hepatocytes. *p < 0.5, **p < 0.01.
MitoSOX-loaded mitochondria treated with antimycin A. The expected increased ROS readout was not altered by preincubation of the mitochondria with Antcin H (Fig. 6D).

Many investigators have shown that APAP induces mitochondrial superoxide (\(O_2^-\)) and NO, which then react to form peroxynitrite (ONOO\(^-\)), which reacts with protein tyrosine (1, 16–18, 21). In addition, others have shown that the increased peroxynitrite formation exclusively in mitochondria in response to p-JNK depends on APAP increasing superoxide production (34). Therefore, as further support for Antcin H suppressing mitochondrial ROS production, we assessed mitochondrial 3-NT levels by Western blot (Fig. 7A) and immunohistochemistry (Fig. 7B). Antcin H inhibited the formation of 3-NT, which is further evidence confirming inhibition of mitochondrial superoxide anion production.

The increased ROS generation is known to sustain the activation of MAPK upstream of JNK. We reported previously (43, 45) that sustained MKK4 (MAP2K) activation

**FIG. 4.** Effects of Antcin H on initial TNF\(\alpha\) receptor signaling in PMH and expression of p-JNK in GalN/TNF\(\alpha\) model. (A) Hepatocytes from wild-type mice were cultured with 20 ng/ml TNF\(\alpha\) and DMSO or Antcin H (100 \(\mu\)M) for 5, 10, and 30 min. Immunoblotting of PMH extracts was performed using antisera against p-IxB\(z\), IxB\(z\), p-JNK, JNK, and GAPDH. (B, C) Mice were pre-treated with Antcin H (50 mg/kg i.p. or DMSO vehicle) and whole liver homogenate (B) or cytoplasm and mitochondria (C) at various times after GalN/TNF\(\alpha\) were immunoblotted for p-JNK and total JNK with GAPDH or PHB1 as a loading control. Bar graphs at right are as in Figure 3 (\(n=3\)). *\(p<0.5\), **\(p<0.01\).

**FIG. 5.** Effect of Antcin H on JNK activity. Cytoplasm containing endogenous p-JNK was prepared 2 h after APAP (300 mg/kg, i.p.). Kinase activity was measured as production of p-c-Jun in the absence of c-Jun substrate (lane 1) or in the absence (lane 2) or presence of JNK inhibitor (SP600124), DMSO vehicle, or Antcin H as indicated. All assays contained ATP (50 \(\mu\)M). The lower panel confirms equal amount of p-JNK and GAPDH in the various assay conditions (representative of \(n=3\) experiments).
FIG. 6. Effect of Antcin H on ROS generation. (A) Antcin H decreases ROS level of hepatocytes in vitro. Three hours after plating hepatocytes and pretreatment with Antcin H for 2 h, PMH were exposed to APAP (10 mM) for 1 h. Hepatocytes were then incubated for 30 min with DHE probe at 37°C and ROS detected by flow cytometry (left panel). "FL2-H" represents the intensity of fluorescence of DHE, and "Counts" is the cell numbers. Right panel shows summary of data from three experiments normalized to control. (*p < 0.05, n = 3 experiments). (B) Mitochondrial superoxide measured by MitoSOX increased after incubation of liver cytoplasm harvested 2 h after APAP (p-JNKc) containing p-JNK with normal mitochondria. Vehicle (DMSO) had no effect on ROS level, while Antcin H in DMSO significantly inhibited mitochondrial ROS production. "no MitoSOX" and "no p-JNK (normal cytoplasm)" are controls. (*p < 0.05 "no p-JNK" vs. "p-JNKc"; #p < 0.05 "p-JNK" vs. "p-JNKc + Antcin H," n = 3 experiments). (C) Fifty micromolar DPPH with different Antcin H concentrations (0, 2.5, 5, 10, 24, 50, 75, 100, 150, 50, and 300 μM) in triplicate were wrapped in aluminum foil and kept at 30°C for 30 min in the dark. All measurements were done under dim light. Spectrophotometric measurements were done at 517 nm. BHA (50, 100, 50, and 300 μM) was used as a positive control. Radical scavenging activity (%) = (OD (Blank) − OD (Sample))/OD (Control) × 100%. (D) Antimycin A inhibition of Complex III generated superoxide from mitochondria. FeTCCP (iron protoporphyrin) effectively inhibited superoxide production, whereas Antcin H did not affect superoxide level. ROS, reactive oxygen species. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars
Antcin H directly disrupts the binding of JNK to mitochondria

The blockade of any step in the sustained JNK activation loop (MAPK cascade → p-JNK → Sab → mitochondrial ROS → MAPK cascade) (45) would limit p-JNK and provide protection against APAP hepatotoxicity. However, since we observed that Antcin H inhibited p-JNK-induced ROS production in isolated mitochondria, we hypothesized that Antcin H interferes with the binding or action of p-JNK on mitochondrial Sab. This interaction activates an intramitochondrial signaling pathway, which inhibits respiration and increases ROS production.

Binding of p-JNK to Sab is the first step in initiating this pathway (42). We examined the mitochondrial Sab protein level by Western blot (Fig. 8A) and found no effect of Antcin H. We treated normal liver mitochondria with cytoplasm containing p-JNK (isolated from 2 h APAP) in the presence of different concentrations of Antcin H (12.5, 50, 100, 300 μM). The washed mitochondria were then analyzed for bound p-JNK and total JNK protein level by immunoblotting. In the presence of Antcin H, both p-JNK and total JNK binding to mitochondria were decreased (Fig. 8B). PHB1 was used as mitochondria protein loading level, while GAPDH was used to assess any cytoplasmic protein contamination.

Since Dexa has a similar structure as Antcin H (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/ars), we compared their effects. Antcin H inhibited p-JNK binding, but Dexa had no effect (Fig. 8C). To confirm the link between Antcin H and p-JNK interaction with mitochondria, we examined the changes of mitochondrial OCR induced by recombinant p-JNK1/2 by using Seahorse XF24 analyzer.

Normal mitochondria were incubated with Antcin H or Dexa for 30 min and then exposed to p-JNK1/2 plus ATP in the presence of glutamate and malate. Neither Antcin H nor Dexa inhibited respiration in the presence of unactivated JNK1/2 plus ATP. However, Antcin H inhibited the impairment of oxidative phosphorylation and maximum respiratory capacity in the presence of p-JNK1/2 plus ATP, whereas Dexa did not (Fig. 8D).

Thus, our findings suggest that Antcin H directly inhibits the binding and effects of p-JNK on mitochondria. This leads to decreased mitochondrial ROS production, which abrogates the effect of mitochondrial ROS to induce MAPK cascade activation of JNK.

Discussion

In preclinical and clinical studies, AC has been shown to have multiple biological activities, including hepatoprotective effects (19, 24, 30). Although the chemistry of AC has been extensively studied (10), the active components that contribute to its biological functions are far from clear. Identification of active ingredients from AC is needed for a better utilization of this medicinal mushroom. Antcin H is a major triterpenoid compound isolated from AC (31), which possesses a favorable pharmacokinetic feature (32).

In the current study, we have demonstrated the hepatoprotective effect of Antcin H using mouse models of both APAP necrotic and GalN/TNFα-induced apoptotic liver injury. Antcin H was still highly effective against APAP-induced liver injury when given after APAP in vivo, suggesting it has therapeutic potential. Because these two liver injury models exhibit JNK dependence while differing in...
FIG. 8. Effects of Antcin H on the interaction between JNK and mitochondria. (A) Western blotting of Sab in mitochondria isolated from control and Antcin H treated alone at different times after APAP treatment. PHB1 is loading control. (B) Isolated liver mitochondria were incubated at 30°C for 1 h with cytoplasm obtained 2 h after APAP (300 mg/kg), with or without Antcin H (preincubated for 30 min). Extracts of washed mitochondria were then immunoblotted for p-JNK, total JNK, and loading control for mitochondria (PHB1) and cytoplasmic contamination (GAPDH). (C) Comparison of the effect of Antcin H and Dexa on p-JNK binding performed as B. Bar graphs in (B, C) represent densitometry of p-JNK/PHB1 (n = 3). *p < 0.05, **p < 0.01. (D) Mitochondria were incubated with Antcin H (25 μM) or Dexa (25 μM) in vehicle for 30 min and OCR measured as described in the Materials and Methods section in Seahorse apparatus in the presence of JNK1/2 plus ATP or p-JNK1/2 plus ATP (upper panel). The data are presented as mean ± SD of five wells for one representative experiment. Lower panel, bar graphs of oxidative phosphorylation and maximal respiration with or without Dexa and Antcin H derived from Seahorse data. Neither Dexa nor Antcin H affected OCR in the absence of p-JNK (Supplementary Fig. S2). Bar graphs summarized the results of three Seahorse experiments (oxidative phosphorylation: OCR with ADP minus oligomycin; Maximal respiration: CCCP minus AA) *p < 0.05. AA, Antimycin A; OCR, oxygen consumption rate. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars
mode of cell death, we explored the effect of Antcin H on the JNK signaling pathway.

JNK can be initially activated by extracellular death receptor signaling (TNF) or by intracellular mitochondrial stress (e.g., APAP). However, sustained JNK activation in cell death scenarios depends upon the binding of p-JNK to and its phosphorylation of Sab, an outer membrane p-JNK "receptor" and substrate. This leads to an intramitochondrial signaling pathway that disrupts electron transport leading to the release of ROS, which continues to activate the MAPK cascade through a self-sustained MLK3/ASK1/p-MKK4/p-JNK/Sab/ROS pathway (43–45). It is currently believed that continued H2O2 release from mitochondria in response to p-JNK interaction with Sab leads to mitochondrial ROS release, which activates MAP3K and inhibits MAPK phosphatases, so JNK activation is sustained (29, 42).

Antcin H inhibited sustained JNK activation and ROS production. We excluded effects on APAP metabolic activation and the possibility that Antcin H is a radical scavenger. In addition, Antcin H did not inhibit antimycin A-induced O2•−, or act as a direct JNK inhibitor.

However, in normal mitochondria, Antcin H inhibited the Sab-dependent p-JNK-mediated inhibition of mitochondrial oxidative phosphorylation and maximum respiratory capacity, as well as the p-JNK-induced mitochondrial O2•−. Since the p-JNK-mediated mitochondrial ROS production is self-sustaining (13), we therefore addressed the possibility that Antcin H might interfere with the binding of p-JNK to mitochondrial Sab, which would block downstream ROS production. Using normal liver mitochondria incubated with cytoplasmic extracts from APAP-treated mice, we found that Antcin H decreased the binding of p-JNK to mitochondria without affecting p-JNK levels during the incubation. This leads us to the conclusion that the protective effect of Antcin H is mediated by inhibition of the binding of p-JNK to Sab (Fig. 9).

There are a few caveats about our work. We cannot at this point definitively conclude that Antcin H binds to Sab (as a competitive or allosteric inhibitor) or if it indirectly causes decreased binding by affecting another mitochondrial protein, which changes the binding of p-JNK to Sab, or by affecting the physicochemical properties of the outer membrane lipids. More work will be required to elucidate this mechanism for inhibition of p-JNK binding to Sab. In addition, more work will be needed on pharmacokinetic properties of Antcin H and on the accumulation of Antcin H on the outer membrane of mitochondria in vivo. In addition, the effect of other Antcin compounds from AC will need to be assessed in future studies.

However, we believe that this is a very novel finding as Antcin H is the first identified compound that can interfere with the binding of p-JNK to mitochondria. This is particularly important since it is not a direct JNK inhibitor and therefore would not be expected to inhibit the protective/beneficial aspects of JNK signaling (2, 40), such as AP-1 transcription factor activation (39, 47). This has been a theoretical limitation in the therapeutic application of JNK inhibitors, particularly in liver injury, because of the potential.

FIG. 9. Mechanism of protection by Antcin H: Inhibition of sustained JNK activation and blocking the binding of p-JNK to mitochondria. The interplay of p-JNK and mitochondrial Sab leads to increased ROS release, which activates the MAPKinase cascade and may inactivate JNK phosphatase (MKP1), leading to a self-perpetuating pathway, which activates mitochondrial ROS production and cytoplastic JNK activation. Thus, the ROS may then amplify the effects of APAP on mitochondria leading to MPT induction and necrosis. In the APAP model, GSH and ATP depletion and a high level of ROS incapacitate caspase activity. Sustained JNK activation also plays an important role in apoptosis by its known actions on c-FLIP degradation, allowing caspase-8 to cleave Bid to t-Bid, as well as activation of Bax and inactivation of Bcl-XL and Mcl-1, thereby promoting MOMP and cytochrome c release. Antcin H disrupts the ability of p-JNK to interact with mitochondrial Sab, thereby abrogating the mechanism for sustained JNK activation. MOMP, mitochondrial outer membrane permeabilization; MPT, mitochondrial permeability transition. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

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for inhibition of liver regeneration and other beneficial effects of transient JNK activation. Therefore, Antcin H may offer a novel therapeutic to prevent or treat liver injury and perhaps other diseases associated with the adverse effects of sustained JNK activation, which require the interaction of JNK and mitochondrial Sab.

Materials and Methods

Chemicals and reagents

Antcin H (purity 95% or higher) was isolated from AC by Ye’s laboratory as reported previously (31). Acetaminophen (APAP), dexamethasone (Dexa), galactosamine (GalN), actinomycin D, ADP, ATP, dihydroethidium, oligomycin, CCCP, FeTCCP, antimycin A, and GAPDH antibody were purchased from Sigma-Aldrich (St. Louis, MO). TNFα was purchased from Calbiochem (Billerica, MA). Antibodies specific for p-JNK, JNK, p-MKK4, MKK4, and β-actin were purchased from Cell Signaling Technology (Denvers, MA). NAPQI adduct antiserum was provided by Laura James at the University of Arkansas. 3-Nitrotyrosine antibody for immunohistochemistry and immunoblotting was obtained from Abcam (Cambridge, MA). Sab antibody was purchased from Santa Cruz Biotechnology (Dallas, TX).

Animal experiments

Male C57BL/6N Hsd mice (6 to 8 weeks of age) were purchased from Harlan Bioproducts for Science Inc. (Indianapolis, IN). All mice were housed in a temperature-, light-, and humidity-controlled environment that is accredited by the Associate for Assessment and Accreditation of Laboratory Animal Care. Mice were maintained on standard laboratory chow, had free access to water, and acclimatized for at least 1 week. APAP was dissolved in 55°C warm PBS. Overnight-fasted mice received APAP 300 mg/kg body weight by intraperitoneal (i.p.) injection as described before (43). Antcin H dissolved in corn oil (to avoid DMSO) was injected i.p. 1 h before or after APAP treatment. Plasma and liver tissue were collected 1, 2, 4, and 24 h after APAP. Overnight-fasted mice received GalN (800 mg/kg)/TNFα (12 μg/kg) dissolved in sterile PBS by i.p. injection as described before (43). Mice were pretreated with Antcin H, 6 and 1 h in DMSO, before GalN/TNFα injection. Plasma and liver tissue were collected 30 min, 1, 2, and 6 h after GalN/TNFα. A portion of the liver was fixed in 10% neutral formalin for histology, and the rest of the tissue was used for subcellular fractionation and immunoblotting. Alanine aminotransferase (ALT) was measured using reagent kits from Teclo Diagnostics (Anaheim, CA).

Immunohistochemical and chemical staining

After deparaffinization and rehydration of neutral buffered formalin-fixed paraffin-embedded tissue sections (5 μm thickness), primary antibody to 3-Nitrotyrosine immunostaining was performed using the Leica BOND Stainer apparatus as described before (43). Liver sections were also stained with hematoxylin and cosin.

Cell culture and treatments

PMHs were isolated from C57BL/6 N mice as previously described (8). The viability of isolated hepatocytes was assessed by trypan blue dye exclusion. The cells used for all experiments had a viability exceeding 88%. Three hours after plating hepatocytes, the medium containing serum and phenol red was replaced with serum-free DMEM/F12 medium containing 100 U/mL penicillin and 0.1 mg/mL streptomycin. After pretreatment with DMSO or Antcin H for 2 h, the medium was replaced with 10 mM APAP dissolved in prewarmed DMEM/F12 medium. Two hours after APAP exposure, the medium was removed and replaced with DMEM/F12 medium supplemented with Antcin H. Cell lysates were collected at 1, 2, or 4 h, and cell death assessed at 24 h by double staining of SYTOX Green (1 μM) and Hoechst 33258 (8 μg/mL) (28).

In other experiments, cultured hepatocytes were incubated with actinomycin D (Act D; 0.5 μg/mL)/TNFα (20 ng/mL) with Antcin H or TNFα with Antcin H. After the indicated time points, cell lysates were stained with Hoechst 33258 for apoptotic counts (9).

GSH measurement

GSH was measured by spectrophotometric/microplate reader using the enzymatic recycling method as previously described (33).

JNK kinase assay measured by c-Jun phosphorylation assay

JNK activity was determined according to the modified protocol described below using the nonradioactive SAPK/JNK Kinase Assay Kit (No. 8794; Cell Signaling Technology). Two hours after APAP 300 mg/kg i.p., liver was homogenized in the isolation buffer supplemented with protease and phosphatase inhibitor cocktail dissolved in DMSO or water. Cytoplasm was obtained after two times centrifugation at 15,000× g, 4°C. Thirty micrograms of cytoplasm was incubated with different concentrations of DMSO, SP600125, or Antcin H at 30°C for 10 min followed by addition of 50 μM ATP and kinase substrate, c-Jun fusion protein (1 μg), and incubated at 30°C for 30 min. Reaction was terminated with SDS sample buffer and boiled for 3 min. Western blot for p-c-Jun, c-Jun, p-JNK, and GAPDH was then performed.

p-JNK and mitochondria binding assay

Liver mitochondria from PBS-treated mouse were isolated as described before (45). Liver cytoplasm from APAP 300 mg/kg i.p.-treated mouse was isolated 2 h after treatment as described before (45). Mitochondria (1 mg/100 μL, 5% BSA) was preincubated at room temperature for 15 min. Cytoplasm (5 mg/600 μL) with Antcin H or structurally similar dexamethasone was preincubated at room temperature for 30 min and then added to mitochondria and incubated for 1 h at room temperature. Mitochondria were then washed in 10 mL of isolation buffer with protease and phosphatase inhibitor cocktail. Mitochondria pellets were dissolved in RIPA buffer for Western blot.

Mitochondrial respiration measurement

Livers were homogenized in mitochondrial assay solution (MAS) (70 mM sucrose, 25 mM mannitol, 10 mM KH2PO4, 5 mM MgCl2, 2 mM HEPES, 1 mM EGTA, pH 7.2 at 37°C) supplemented with pyruvate and malate, without protease.
and phosphatase inhibitors. Mitochondria were isolated by differential centrifugation and resuspended in MAS.

Fifty nanograms (160 U/mg) activated or unactivated JNK was diluted in 25 μl of MAS and incubated with Antcin H or dexamethasone (Dexa) at room temperature for 30 min. Five micrograms of mitochondria was diluted in 24 μl MAS supplemented with ATP 6 μM and then JNK was added to with Antcin H or Dexa. Fifty microliters premixed mitochondria 5 μg and 50 ng JNK with 24–50 μM Antcin H or Dexa was then loaded into each well of Seahorse XF24 analyzer cell culture microplate on ice and was centrifuged at 3000 g and 5 min at 8°C. The resulting supernatant was then loaded into each well of Seahorse XF24 analyzer cell culture microplate on ice and was centrifuged at 3000 g for 5 min at 8°C, and then incubated at 37°C in CO2-free incubator for 15 min.

After incubation, each well was fed with 450 μl of 37°C prewarmed MAS supplemented with substrate, and oxygen consumption rate (OCR) was measured. The program was set to equilibrate at 37°C for 5 min before sequential measurement of basal OCR (State 2 respiration), followed by injection of ADP (4 mM; final), oligomycin (2.5 μg/ml; final), CCCP (4 μM; final), and antimycin A (4 μM; final), and OCR was measured. After ADP injection, mitochondrial oxidative phosphorylation (State 3 respiration) was determined. OCR after oligomycin injection was referred to as State 4 respiration, and CCCP induced OCR as maximal respiratory capacity (37, 42, 45).

Subcellular fractionation for cytoplasm and mitochondria from liver

Livers were homogenized in ice cold homogenizing buffer (250 mM sucrose, 10 mM Tris, 2 mM EGTA, pH 7.4) supplemented with protease and phosphatases inhibitor cocktail. The homogenate was centrifuged at 1000 x g for 5 min; the centrifugation was repeated again for 10 min. The supernatant was centrifuged at 9000 g 10 min. The resulting supernatant is referred to as cytoplasm. The mitochondria pellet was washed with isolation buffer and recentrifuged. The mitochondria were resuspended in MAS and phosphatase inhibitors. Mitochondria were isolated by differential centrifugation and resuspended in MAS.

Western blotting

Thirty micrograms of protein samples was denatured, and SDS-PAGE electrophoresis was performed in Bio-Rad 7.5%, 10%, or 4%–20% Mini-PROTEAN® TGX™ Precast Gel. After transfer to 0.2 μm nitrocellulose membrane using iBlot transfer device (Invitrogen), immunoblot was performed using specific antibodies as described.

Measurement of antimycin A and p-JNK-induced mitochondrial ROS

Mitochondria from C57BL/6 N mice liver were isolated in respiration buffer (MAS buffer supplemented with pyruvate and malate without protease and phosphatase inhibitors). Twenty micrograms of mitochondria in 50 μl of respiration buffer was loaded into precooled 96-well solid bottom Corning Costar plate (Cat. No. 3917) on ice. One hundred microliters of ice cold respiration buffer was supplemented with MitoSOX (2 μM; final), antimycin A (5 μM; final), and ADP (4 μM; final). Antcin H (25 μM; final) or FeTCCP (1 μM; final) was added into indicated wells on ice just before loading into 37°C prewarmed FLUOstar Omega Microplate Reader. Six minutes after equilibration at 37°C in a dark chamber, basal level of fluorescence intensity was measured at Ex355nm/Em590nm and fluorescence intensity was measured at 6-min intervals.

Mitochondria and cytoplasm from APAP 2 h-treated mice or untreated mice were isolated as described above. Twenty micrograms of control mitochondria in 50 μl of respiration buffer was loaded into precooled 96-well plates on ice. MitoSOX (2 μM; final), ADP (4 μM; final), and 100 μg APAP-treated cytoplasm, which provided activated JNK or normal cytoplasm, were added into indicated wells on ice as above and then fluorescence intensity was measured.

Statistical analysis

Data are presented as mean ± SD. T-test was used for comparison of two groups. p < 0.05 was considered statistically significant.

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Author Disclosure Statement

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**Abbreviations Used**

3-NT = 3-Nitrotyrosine  
AC = Antrodia Camphorate  
Act D = actinomycin D  
ALT = alanine aminotransferase  
APAP = acetaminophen  
BHA = butyl hydroxy anisd  
CCCP = carbonyl cyanide 3-chlorophenylhydrazone  
Dexa = dexamethasone  
DMEM = Dulbecco’s modified Eagle’s medium  
DPPH = 2,2-diphenyl-1-picrylhydrazyl  
GalN = galactosamine  
GSH = glutathione  
JNK = c-Jun-N-terminal kinase  
MAS = mitochondrial assay solution  
MKK4 = mitogen-activated protein kinase kinase 4  
NAPQI = N-acetyl-p-benzoquinone  
OCR = oxygen consumption rate  
PBS = phosphate-buffered saline  
PHB1 = prohibitin  
PMH = primary mouse hepatocytes  
RIPA = radioimmunoprecipitation  
ROS = reactive oxygen species  
Sab = Src homology 3 domain-binding protein 5  
TNF = tumor necrosis factor