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Therapeutic potential of carfilzomib, an irreversible proteasome inhibitor, against acetaminophen-induced hepatotoxicity in mice

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Abstract

Overdose of acetaminophen (APAP) is often associated with hepatotoxicity. Carfilzomib (CFZ) shows multiple pharmacological activities including anti-inflammatory potential. Therefore, this study was undertaken to evaluate the possible therapeutic effects of CFZ against APAP-induced hepatotoxicity. Hepatotoxicity was induced by administration of APAP (350 mg/kg, intraperitoneal). Mice were given CFZ (0.125, 0.25, or 0.5 mg/kg, intraperitoneal) 1.5 h after APAP administration. Animals were sacrificed on 6 h and blood and liver tissue samples were collected for analysis. In CFZ-post-treated group, there was significant and dose-dependent decrease in serum alanine aminotransferase levels. The level of tumor necrosis factor-α (TNF-α), reactive oxygen species, and NO decreased, whereas glutathione increased significantly by CFZ post-treatment. Upregulated mRNA expression of COX-II and iNOS were significantly downregulated by CFZ post-treatment. CFZ may exert its hepatoprotective action by alleviating inflammatory, oxidative, and nitrosative stress via inhibition of TNF-α, COX-II, and iNOS.

KEYWORDS

acetaminophen, carfilzomib, hepatotoxicity, oxidative and nitrosative stress, TNF-α, COX-II and iNOS

1 | INTRODUCTION

Acute overdose of acetaminophen (APAP) causes fatal hepatotoxicity.[1] The hepatotoxicity of APAP is closely linked to its metabolism. Metabolism by the cytochrome P450 system produces a metabolite, N-acetyl-p-benzoquinone imine (NAPQI) that is directly toxic to the liver.[2] When APAP is taken in therapeutic doses, this potentially dangerous metabolite is rendered harmless by reduced glutathione (GSH). However, when APAP is taken in excess, the amount and rate of formation of NAPQI are greatly increased; depleting the liver GSH store,[3] APAP hepatotoxicity is characterized by greatly increased oxidative stress due to mitochondrial dysfunction and the generation of superoxide.[4] In addition to the generation of reactive oxygen species (ROS), APAP also causes the production of reactive nitrogen species (RNS) leading to nitrotyrosine adduction of proteins.[5]

Initial hepatocellular damage caused by NAPQI can lead to the release of damage-associated molecular patterns such as high-mobility group box 1 protein, heat shock proteins, DNA fragments, and others [6] which can directly activate non-parenchymal hepatic cells, Kupffer cells, and other innate immune cells.[7,8] Activated cells can release a wide range of inflammatory mediators, such as ROS and RNS,[9] proinflammatory cytokines,[6] amplifying the oxidative stress and inflammatory responses, consequently promoting further hepatic injury.[10] Moreover, the excessive ROS and RNS production during inflammation contributes to the depletion of GSH,[11] which could result in further NAPQI accumulation. Neutrophil and macrophage accumulation in the liver has also been associated with APAP-induced liver injury [12,13] and several other experimental animal models of liver injury such as ischemia-reperfusion injury [14] and endotoxemia.[15] Neutrophils and macrophages under certain circumstances can severely aggravate tissue damage. In APAP-induced hepatotoxicity, excessive neutrophil and macrophage activity can contribute to liver inflammation and injury.[12,13]

Proteasome inhibitors have been demonstrated to be beneficial in several pathologic conditions, including autoimmune disorders in animal models and cancers in human.[16,17] Proteasome inhibitors are also shown to have organ-protective effects in experimental ischemia-reperfusion injury of the brain, heart, and kidney.[12] Blockage of NF-κB activation is thought to account for the majority of protective

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effects by proteasome inhibition. Carfilzomib (CFZ) is a novel, well tolerated, irreversible proteasome inhibitor with minimal side effects.[18] Importantly, proteasome inhibitors can overcome NF-κB activation by inhibiting degradation of IkB and the P105 precursor of p50 subunit of NF-κB.[19] This results in decreasing inflammatory mediators including TNFα, COX-II, and iNOS each of which has been implicated in hepatotoxicity. In a recent study, it has been demonstrated that CFZ protects against diethyl nitrosamine-induced hepatotoxicity in rats.[20] Concerning APAP hepatotoxicity, we hypothesized that CFZ could inhibit the activation of NF-κB and associated inflammatory mediators, alleviate oxidative stress in the liver and reduce hepatocyte death. To provide evidence for these predictions, we designed an animal study to investigate the effect of CFZ on APAP-induced injury in the mice liver.

2 | MATERIALS AND METHODS

2.1 | Chemicals

APAP, Ellman's reagent, NADPH, sulfanilamide, sodium nitrate, 2′,7′-dichlorodihydrofluorescein diacetate, vanadium trichloride, DMSO, and N-(naphthyl)-ethylenediamine dihydrochloride were obtained from Sigma (St. Louis, MO). CFZ was obtained from Active Biochem (Hong Kong, China). Diagnostic kits for measurement of alanine aminotransferase (ALT) and total protein were obtained from Randox Laboratories Ltd. (Manchester, United Kingdom). Diagnostic ELISA kit for measurement of tumor necrosis factor-alpha (TNF-α) was obtained from R&D systems (Minneapolis, MN). TRIzol reagent was purchased from Invitrogen Company (Grand Island, NY). High Capacity cDNA Reverse Transcription kit and SYBR® Green PCR Master Mix were purchased from Applied Biosystems (Foster City, CA). Primers for mice COX-II, iNOS, and GAPDH were purchased from Integrated DNA Technologies (IDT; Coralville, IA). All other chemicals were of highest analytical grade.

2.2 | Animals

Male CD-1 mice weighing 25–30 g were used in all experiments. They were obtained from the Experimental Animal Care Center, King Saud University, Riyadh, Saudi Arabia. All animals were fasted 18 h before the experiments to sensitize mice to APAP toxicity.

2.3 | Experimental protocol

To evaluate the effect of different doses of CFZ on APAP-induced hepatotoxicity in mice, animals were divided randomly into eight groups; control group, CFZ groups (0.125, 0.25, and 0.5 mg/kg intraperitoneal; i.p.), APAP group (350 mg/kg i.p.) and APAP (350 mg/kg i.p.) plus CFZ groups (0.125, 0.25 and 0.5 mg/kg i.p.). CFZ was administered in 100 μL of DMSO/kg. Control and APAP groups received the same amount of DMSO. Animals were sacrificed 6 h after APAP injection. Sera were isolated for measurements of ALT, a specific marker of liver injury.

To explore the possible mechanisms of CFZ against APAP-induced hepatotoxicity, mice were randomly allocated into four groups: control group, CFZ group (0.5 mg/kg), APAP group (350 mg/kg), and APAP (350 mg/kg) plus CFZ (0.5 mg/kg) group. CFZ administered 1.5 h after APAP. Animals were sacrificed 6 h after APAP injection.

2.4 | Serum and tissue preparation

Blood samples were collected from the orbital venous plexus, under light ether anesthesia and sera were separated by centrifugation for 5 min at 1000×*g*. Animals were sacrificed by cervical dislocation and the livers were quickly isolated and weighed. Homogenization was carried out in saline to yield 10% (w/v) tissue homogenates.

2.5 | Biochemical analysis

Serum ALT activities were determined according to the method of Bergmeyer.[21] GSH was determined according to the method of Ellman.[22] Total nitrate/nitrite (NOx) in liver tissues was measured as nitrite according to the method of Miranda et al.[23] Tissue ROS levels were determined according to the method of Noh et al.[24] Levels of TNF-α in serum were measured using ELISA kits (R&D) according to the manufacturer's instructions. Protein contents were determined by the biuret reaction using bovine serum albumin as standard.

2.6 | Total RNA extraction and cDNA synthesis

Total cellular RNA from liver tissues was isolated using TRIzol reagent according to the manufacturer's instructions. Thereafter, first strand cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit, according to the manufacturer's instructions.

2.7 | Quantification of mRNA expression by real-time PCR (RT-PCR)

Quantitative analysis of specific mRNA expression was performed by RT-PCR by subjecting the resulting cDNA to PCR amplification using 96-well optical reaction plates in the Quant Studio 6 Flex Fast RT-PCR System (Applied Biosystems). Mice primers were used with the following sequences: COX-II, forward: 5′-CATTCTTTGCCCAGCACTTCAC-3′ and reverse: 5′-GACCAGGCACCAGACCAAAGAC-3′, iNOS, forward: 5′-CCTGGTACGGGCATTGCT-3′ and reverse: 5′-GCTCATGCGGCCTCCTTT-3′, GAPDH, forward: 5′-TGAAGCAGGCATCTGAGGG-3′ and reverse: 5′-CGAAGGTGGAAGAGTGGGAG-3′. The RT-PCR data were analyzed using the relative gene expression method.

2.8 | Statistical analysis

Data are expressed as means ± SD. Statistical comparison between different groups were done using one-way analysis of variance followed by Tukey–Kramer multiple comparisons test. Significance was accepted at $P < 0.05$.

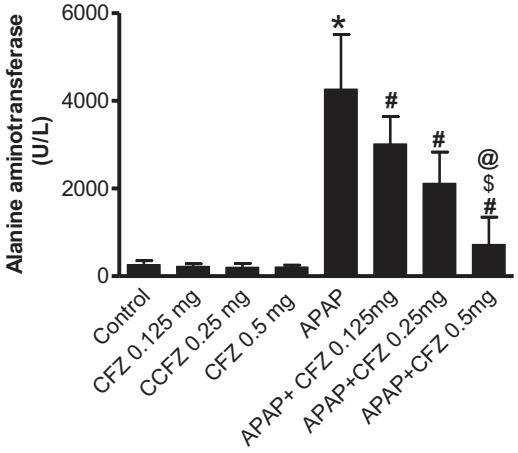


FIGURE 1 Effect of CFZ, APAP, and their combination on serum ALT activity. Mice were given different doses of CFZ (0.125, 0.25, and 0.5 mg/kg i.p.) 1.5 h after APAP administration (350 mg/kg i.p.). Blood was withdrawn 6 h after injection of APAP and serum ALT activity was assayed. Each group represents the means ± SD of six mice. *Significantly different from control. #Significantly different from APAP. @Significantly different from APAP plus CFZ (0.125 mg) or APAP plus CFZ (0.25 mg). $Non-significantly different from control.

3 | RESULTS

3.1 | Effect of CFZ, APAP, and their combination on serum ALT activity

Figure 1 shows the effect of acute administration of APAP on ALT activity in CFZ treated and untreated mice. Administration of single dose of APAP (350 mg/kg, i.p.) resulted in a significant increase in the level of ALT activity as compared with the control group (260 ± 37 U/L for control versus 4265 ± 510 U/L for APAP). Administration of CFZ (0.125, 0.25, or 0.5 mg/kg i.p.) 1.5 h after APAP showed a significant decrease in ALT activity as compared with the APAP group (3015 ± 255, 2115 ± 292, or 724 ± 25 U/L for APAP + CFZ versus 4265 ± 510 U/L for APAP). Administration of CFZ alone showed non-significant changes in serum ALT activity as compared with the control group. It should be noted that administration of CFZ (1 mg/kg i.p.) 1.5 h after APAP produced no significant difference from the administration CFZ (0.5 mg/kg i.p.) 1.5 h after APAP.

3.2 | Effect of CFZ, APAP, and their combination on serum TNF-α and hepatic GSH, ROS, and NOx

Figures 2A–2D show, respectively, the effect of acute administration of APAP on hepatic TNF-α GSH, ROS and NOx, and in CFZ treated and untreated mice. Administration of single dose of APAP (350 mg/kg, i.p.) resulted in 350% increase in the levels TNF-α a significant 45% decrease in the level of hepatic GSH, 41% increase in the level of ROS, and 80% increase in the level of NOx as compared with the control group. Administration of CFZ (0.5 mg/kg) 1.5 h after APAP showed a reversal of APAP-induced changes toward the control values. Administration of CFZ alone showed non-significant changes in hepatic GSH, ROS, NOx, and TNF-α as compared with the control group.

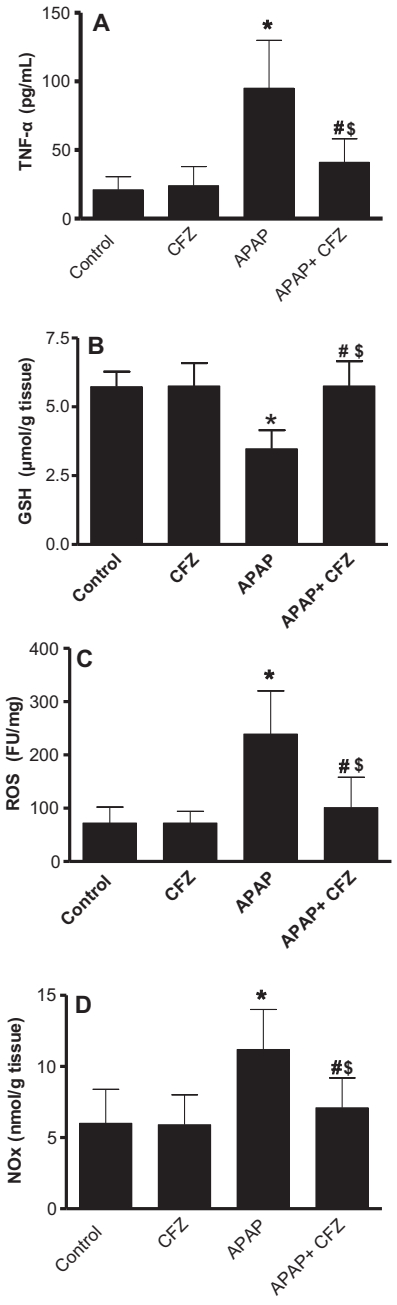


FIGURE 2 Effect of CFZ, APAP, and their combination on serum TNF-alpha and hepatic oxidative and nitrosative stress parameters. Mice were given CFZ (0.5 mg/kg i.p.) 1.5 h after APAP administration (350 mg/kg i.p.). Livers were isolated 6 h after injection of APAP and hepatic oxidative and nitrosative stress parameters were assayed as described in section *Materials and Methods*. Each group represents the means ± SD of six mice. *Significantly different from control. #Significantly different from APAP. $Non-significantly different from control.

3.3 | Effect of CFZ, APAP, and their combination on hepatic COX-II and iNOS gene expression

Figures 3A and 3B show the effect of acute administration of APAP on hepatic iNOS and COX-II gene expression in CFZ treated and untreated mice. Administration of single dose of APAP (350 mg/kg, i.p.) resulted in a ninefold increase in the level of iNOS and 15-fold

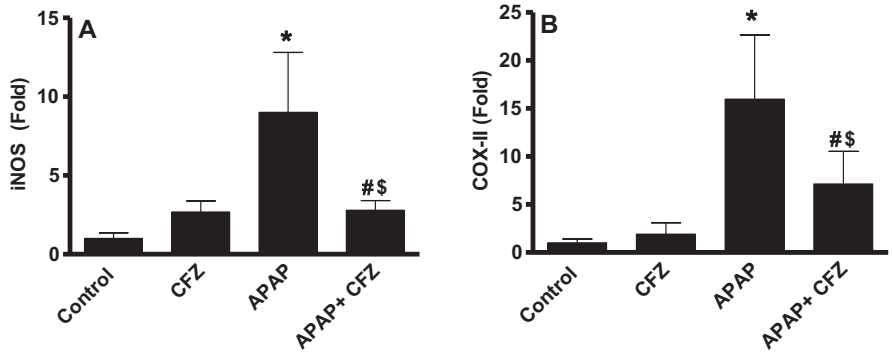


FIGURE 3 Effect of CFZ, APAP, and their combination on hepatic gene expression of iNOS and COX-II. Mice were given CFZ (0.5 mg/kg i.p.) 1.5 h after APAP administration (350 mg/kg i.p.). Livers were isolated 6 h after injection of APAP and hepatic gene expression was assayed as described in section *Materials and Methods*. Each group represents the means ± SD of six mice. *Significantly different from control. #Significantly different from APAP. $Non-significantly different from control.

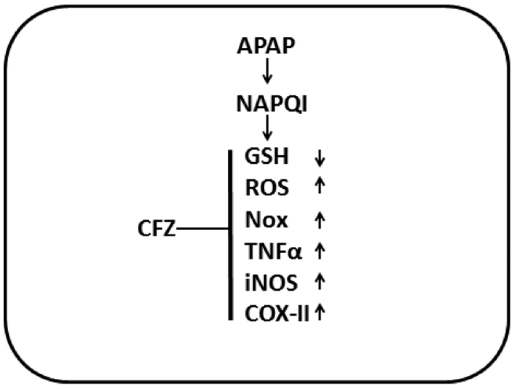


FIGURE 4 Diagrammatic representation of the possible mechanisms involved in APAP-induced hepatocyte death and its suggested points of intervention by CFZ.

increase in the level of hepatic COX-2 as compared with the control group. Administration of CFZ (0.5 mg/kg) 1.5 h after APAP showed a reversal of APAP-induced changes toward the control values. Administration of CFZ alone showed non-significant changes in hepatic iNOS and COX-II as compared with the control group.

4 | DISCUSSION

This study uses for the first time CFZ as a therapeutic agent in hepatotoxicity caused by APAP. Post-treatment with CFZ was found to decrease serum ALT levels in a dose-dependent manner. Thus, considering that no study has focused on the mechanisms by which CFZ exerts its effect on APAP-induced hepatotoxicity, this study was designed to evaluate the benefits of the CFZ and open new perspectives for its application.

TNF-α has been demonstrated to play a role in the pathogenesis of APAP-induced liver injury.[25] Meanwhile, TNF-α can induce other cytokine production.[26] These cytokines amplify the inflammatory response. Furthermore, previous reports demonstrated that inhibition of TNF-α could alleviate liver injury [27] in agreement with our results. Post-treatment with CFZ was found to produce significantly more GSH than APAP-treated mice. Our results agree with the other reports pertaining to APAP induced GSH depletion after APAP intoxication. Previous studies on the mechanisms of APAP-induced hepatotoxicity have shown that GSH plays a key role in the detoxification of the reactive toxic metabolites of APAP, and that liver necrosis begins when GSH stores are markedly depleted.[3] It appears to be fairly will accepted that hepatotoxicity of APAP is associated with the generation of free radicals.[28] The enhanced production of liver ROS observed in our study is in agreement with other studies.[29] Post-treatment with CFZ was found to produce significantly less ROS than APAP-treated mice. Thus, it may be concluded that CFZ is capable of decreasing free radicals generated by APAP administration probably by increasing the antioxidant enzymes.[30–32] Our data strongly suggest the importance of NO in the pathogenesis of APAP-induced hepatotoxicity, in agreement with other studies.[33,34] Post-treatment with CFZ was found to produce significantly less NO than APAP-treated mice. Our data strongly suggest that CFZ inhibits the iNOS. Aminoguanidine, a relatively iNOS inhibitor, also reduces APAP-induced hepatotoxicity.[33]

NF-κB regulates the expression of genes that control inflammatory mediators including iNOS II and COX-II, each of which has been implicated in hepatotoxicity.[35] Activation of a transcription factor NFκB is strongly implicated in APAP toxicity.[36] Increased activation of NFκB was observed in mice macrophages treated with APAP.[37] Substances such as curcumin and silymarin protect the liver against damage via inhibition of NF-κB.[38,39] In our study, there was upregulation in hepatic iNOS as well as COX-II in the APAP treated group, whereas CFZ administration inhibited iNOS and COX-II upregulation, probably via inhibition of NF-κB, thereby alleviating hepatic inflammation and damage.

Taken together, CFZ may exert its hepatoprotective action by alleviating inflammatory and oxido-nitrosative stress via inhibition of TNF-α, COX-II, and iNOS (Figure 4) and could be a potential candidate for the treatment of APAP-induced hepatotoxicity.

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