A Possible Antineoplastic Potential of Selective, Irreversible Proteasome Inhibitor, Carfilzomib on Chemically Induced Hepatocarcinogenesis in Rats

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ABSTRACT: The antineoplastic effect of carfilzomib (CFZ) against chemically induced hepatocarcinogenesis was studied. A total of 60 male Wistar albino rats were divided into six groups with 10 animals in each group. Rats in group 1 (control group) were given dimethylsulphoxide (DMSO) (0.4 mL/kg i.p) twice a week for 3 weeks from week 8 to week 10. Animals in groups 2 and 3 were given CFZ (2 and 4 mg/kg i.p) twice a week from week 8 to week 10, respectively. Rats in group 4 were given diethylnitrosamine (DENA) at a dose of 0.01% in drinking water for 10 weeks and received a DMSO (0.4 mL/kg i.p) twice a week from week 8 to week 10. Animals in groups 5 and 6 were given DENA at a dose of 0.01% in drinking water for 10 weeks and treated with CFZ (2 and 4 mg/kg i.p) twice a week from week 8 to week 10, respectively. CFZ succeeded in suppressing the elevated serum tumor marker α-fetoprotein and carcinoembryonic antigen. The antineoplastic effect of CFZ was also accompanied by normalization of elevated hepatic tissue growth factors, matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-1, and augmentation of hepatic endostatin and metallothionein. A histopathological examination of liver samples treated with CFZ after DENA intoxication correlated with the biochemical observation. Treatment with CFZ confers an antineoplastic activity against chemically induced hepatocarcinogenesis. These findings suggest that CFZ plays a pivotal role in the treatment of hepatocarcinogenesis.

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KEYWORDS: Proteasome Inhibitor; Carfilzomib; Hepatocellular Carcinogenesis

INTRODUCTION

The ubiquitin proteasome pathway is responsible for the degradation of the majority of regulatory proteins in eukaryotic cells, including proteins that control apoptosis, cell cycle progression, and DNA repair and for that reason it plays a critical role in preserving normal cellular homeostasis [1]. Inhibition of the proteasome leads to stabilization and accumulation of these proteasome substrates, resulting in concomitant activation of pro- and antiproliferative signals, disruption of cell cycle regulation, and, ultimately, activation of apoptotic pathways and cell death [1, 2].

Carfilzomib (CFZ) is a highly interesting compound, which can provide a high response in the case of multiple myeloma than that of bortezomib [3]. CFZ (previously known as PR-171) is a tetrapeptide epoxyketone-based irreversible proteasome inhibitor. As an irreversible inhibitor, CFZ produces more sustained inhibition of the proteasome compared with bortezomib because the synthesis of new proteasome complexes is required to reverse the effects of CFZ [4]. Compared with bortezomib, CFZ is a more potent and more selective inhibitor of the chymotrypsin-like activity of the proteasome and the immunoproteasome [5].
The liver is the unique organ for studying chemical carcinogenesis in vivo during the early stages of initiated cells as altered foci. It is a frequent site for the development of chemically induced cancer in rodents [6]. Experimental hepatocarcinogenesis can be induced by various chemical carcinogens, such as diethylnitrosamine (DENA), 2-acetylaminofluorene, and aflatoxin B1.

To investigate a possible role of CFZ in liver carcinogenesis, we used the DENA rat carcinogenesis model. DENA is a potent hepatocarcinogenic nitrosamine present in tobacco smoke, water, cheddar cheese, cured and fried meals, occupational settings, cosmetics, agricultural chemicals, and pharmaceutical agents [7–10]. It has been suggested that, on metabolic activation, it produces the promutagenic products, O-6-ethyl deoxy guanosine and O-4 and O-6-ethyl deoxy thymidine in liver, which are responsible for its carcinogenic effects [11]. After its use, many studies have showed a series of microscopic lesions called “foci” and “nodules,” which have been designated “preneoplastc” or “premalignant” lesions [12]. During the process of neoplastic transformation, various histochemical and biochemical marker enzymes and protein antigens are expressed depending upon the stages and magnitude of neoplastic lesions. These markers are frequently considered as surrogate end-point biomarkers in a rat liver carcinogenesis model [13].

To date, there are no published studies investigating the role and the possible mechanism of CFZ on chemical hepatocarcinogenesis. Therefore, the present work was undertaken to investigate the possible antineoplastic potential of selective irreversible proteasome inhibitors using CFZ against preneoplastic lesions induced chemically by DENA intoxication.

MATERIALS AND METHODS

Chemicals

DENA will be obtained from Santa Cruz Biotechnology (Santa Cruz, CA). CFZ will be obtained from Active Biochem (Redan, GA). It was freshly dissolved in dimethylsulphoxide (DMSO) prior to injection. Matrix metalloproteinase-2 (MMP-2), tissue inhibitor of metalloproteinase-1 (TIMP-1), vascular endothelial growth factor (VEGF), transforming growth factor-β1 (TGF-β1), and basic fibroblast growth factor (FGF) were purchased from R&D Systems (Minneapolis, MN). Endostatin, metallothionein (MT), carcinoembryonic antigen (CEA), and α-fetoprotein (AFP) were obtained from Usclone Science & Technology (Gunguguoji, China). All other chemicals used were obtained from Sigma (St. Louis, MO, USA) and were of high analytical grade.

Animals

Adult male albino rats of the Wistar strain (170–200 gm) were obtained from Animal Care Center, College of Pharmacy, King Saud University, Riyadh, Kingdom of Saudi Arabia. The animals were housed in cages under standard controlled environmental hygienic conditions (25°C and a 12-h light/dark cycle). Animals have free access to pulverized standard rat pellet diet and fed chow spruce and water ad libitum. The protocol of this study has been approved by Research Ethics Committee of College of Pharmacy, and this study has been adopted by the Committee on Animal Research of King Saud University, Riyadh, Kingdom of Saudi Arabia. All animal procedures followed the international guidelines of proper experimental animal handling.

Experimental Protocol

The animals were divided randomly into six groups, 10 animals in each group. The first group (control) received vehicles used for CFZ, DMSO (0.4 mL/kg i.p), twice a week from week 8 to week 10. The second and third groups received CFZ (2 and 4 mg/kg i.p) twice a week from week 8 to week 10, respectively [5]. The fourth group was given DENA at a dose of 0.01% in drinking water for 10 weeks, and the calculated dose was based on the average daily intake of water for each rat and received a DMSO (0.4 mL/kg i.p) twice a week from week 8 to week 10 [14]. The last two groups were given DENA at a dose of 0.01% in drinking water for 10 weeks [14] and treated with CFZ (2 and 4 mg/kg i.p) twice a week from week 8 to week 10, respectively [5]. The selected concentrations of the drugs and the schedule of dose administration were chosen as guided by our own preliminary experiments. At the end of treatment protocol, the blood samples were taken by cardiac puncture, under light ether anesthesia, into nonheparinized tubes. Serum was separated by centrifugation for 5 min at 1000 × g and stored at –20°C until analysis. Animals were sacrificed by cervical dislocation and the liver was quickly isolated, washed with saline, blotted dry on filter paper, and weighed. A 10% (w/v) homogenate of the liver tissues was prepared in ice-cold saline using a Branson sonifier (250, VWR Scientific, Danbury, CT).

Biochemical Parameters

Enzyme linked immunosorbent assay (ELISA) of AFP and CEA: Quantitative estimation of hepatic tumor
markers AFP and CEA was based on ELISA using assay kits from Uscnlife Science & Technology according to manufacturer’s instructions.

ELISA of different growth factors MMP-2 and TIMP-1, endostatin, and MT levels in liver homogenates: VEGF, FGF, TGF-β1, MMP-2, and TIMP-1 were assayed in the liver homogenates by ELISA using assay kits from R&D Systems according to manufacturer’s instructions. In addition, endostatin and MT were also analyzed by ELISA using assay kits from Usnlife Science & Technology according to manufacturer’s instructions.

**Histopathological Examination**

A histopathological examination was performed on liver sections from the each group. Liver specimens from the each group were removed and a portion of the specimen was fixed in 10% formalin and embedded in paraffin wax. Sections were cut at 4 µm thickness, stained with hematoxylin and eosin, and viewed under light microscope for the investigation of histological changes. To avoid any type of bias, the slides were coded and examined blindly by two experienced histopathologists for the presence of hepatic cirrhosis, hepatocellular dysplasia (dysplastic cirrhotic nodules). In addition, a scoring system was established to evaluate the presence or absence of inflammation and necrosis in the liver sections. Necrosis was scored as 1+ if present and 0 if not detected. The hepatic inflammation was given a score of 0 if absent, 1+ if mild (inflammatory cells present in one high microscopic field only), 2+ or moderate if inflammatory cells are detected in two high power microscopic fields, and 3+ or severe if inflammatory cells are present in three fields or more.

**Statistical Analysis**

Data are expressed as means ± SEM. Statistical comparison between different groups were done using one-way analysis of variance followed by the Tukey–Kramer multiple comparison test to judge the difference between various groups. Significance was accepted at \( P < 0.05 \).

**RESULTS**

**Effect of CFZ Treatment on the Elevated Serum Tumor Markers Levels Induced by Carcinogen Intoxication**

The treatment of normal rats with different doses of CFZ (2 and 4 mg/kg i.p) twice a week for 3 weeks from week 8 to week 10 did not significantly affect the measured serum tumor markers as compared with their control values (Table 1). However, oral administration of DENA (0.01%) in drinking water for 10 weeks induced a significant four- and two-fold increment in both serum tumor markers AFP and CEA, respectively, as compared with their control groups.

The treatment of hepatocarcinogenesis induced by carcinogen intoxication with the chosen doses of CFZ (2 and 4 mg/kg i.p) twice a week for the last 3 weeks from week 8 to week 10 resulted in normalization in the serum level of both selected serum tumor markers (Table 1). A similar effect induced by both doses of CFZ 2 and 4 mg/kg has been observed on different serum tumor markers levels.

**Effects of CFZ Administration on Hepatic Growth Factors: VEGF, TGF-β1, and FGF**

The treatment of normal rats with CFZ (2 and 4 mg/kg i.p) twice a week for 3 weeks from week 8 to week 10 showed nonsignificant changes in the selected hepatic growth factors VEGF, TGF-β1, and FGF as compared with their respective control values (Table 2).

Long-term administration of selected dose of DENA 0.01% in drinking water induced 1.7-, 2.5-, and 3.2-fold increase in hepatic VEGF, TGF-β1, and FGF, respectively, as compared with their corresponding control values. The treatment with the selected doses of CFZ (2 and 4 mg/kg i.p) twice a week for the last 3 weeks induced a profound reduction of hepatic VEGF level as compared with the DENA group (Table 2). Moreover, CFZ treatment protocol provoked a pronounced inhibition of FGF by ca. 60 and 58%, respectively, compared to the control group (Table 2). No tendency of the dose-related effect has been observed for the selected doses of CFZ on VEGF and FGF levels. Likewise, selected doses of CFZ treatment prevent the rise in the hepatic TGF-B1 level as compared to respective control values (Table 2). The effect of CFZ (2 and 4 mg/kg i.p) twice a week is not statistically significant compared to their control values (Table 2). However, oral administration of DENA (0.01%) in drinking water for 10 weeks induced a significant four- and two-fold increment in both serum tumor markers AFP and CEA, respectively, as compared with their control groups.

**TABLE 1. Effects of CFZ Treatment for 3 weeks on the Changes in Serum AFP and CEA Induced by DENA Intoxication**

<table>
<thead>
<tr>
<th>Groups</th>
<th>AFP (ng/mL)</th>
<th>CEA (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.42 ± 0.36</td>
<td>1.24 ± 0.06</td>
</tr>
<tr>
<td>Carfilzomib (2 mg/kg)</td>
<td>5.18 ± 0.30</td>
<td>1.23 ± 0.08</td>
</tr>
<tr>
<td>Carfilzomib (4 mg/kg)</td>
<td>5.55 ± 0.19</td>
<td>1.11 ± 0.1</td>
</tr>
<tr>
<td>DENA 0.01% 10 weeks</td>
<td>23.53 ± 2.28a</td>
<td>2.41 ± 0.1a</td>
</tr>
<tr>
<td>DENA + carfilzomib (2 mg/kg)</td>
<td>7.54 ± 0.56b</td>
<td>1.53 ± 0.13b</td>
</tr>
<tr>
<td>DENA + carfilzomib (4 mg/kg)</td>
<td>7.17 ± 0.59b</td>
<td>1.43 ± 0.16b</td>
</tr>
</tbody>
</table>

All data represent mean values ± SEM (\( n = 10 \)).
aSignificant difference from the control group.
bSignificant difference from the DENA group.
\( P < 0.05 \).
Table 2. Effects of CFZ Treatment for 3 weeks on the Changes in Rat Hepatic Growth Factors, VEGF, TGF-β1, and FGF, Induced by DENA Intoxication

<table>
<thead>
<tr>
<th>Groups</th>
<th>VEGF (pg/mL)</th>
<th>TGF-β1 (pg/mL)</th>
<th>FGF (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>139.93 ± 3.61</td>
<td>937.14 ± 47.38</td>
<td>330.36 ± 24.18</td>
</tr>
<tr>
<td>Carfilzomib (2 mg/kg)</td>
<td>145.2 ± 2.9</td>
<td>833.6 ± 39.37</td>
<td>212.7 ± 17.89</td>
</tr>
<tr>
<td>Carfilzomib (4 mg/kg)</td>
<td>138.6 ± 4.76</td>
<td>893.6 ± 78.28</td>
<td>222.6 ± 20.53</td>
</tr>
<tr>
<td>DENA 0.01% 10 weeks</td>
<td>238.33 ± 11.52</td>
<td>2372.62 ± 151.37</td>
<td>1055.1 ± 48.1</td>
</tr>
<tr>
<td>DENA + carfilzomib (2 mg/kg)</td>
<td>155.61 ± 7.65</td>
<td>958.4 ± 94.8</td>
<td>430.71 ± 26.85</td>
</tr>
<tr>
<td>DENA + carfilzomib (4 mg/kg)</td>
<td>156.89 ± 8.9b</td>
<td>784 ± 71.17b</td>
<td>451.7 ± 39.72</td>
</tr>
</tbody>
</table>

All data represent mean values ± SEM (n = 10).

Table 3. Effects of CFZ Treatment for 3 weeks on the Changes in Rat Hepatic MMP-2 and TIMP-1 Induced by DENA Intoxication

<table>
<thead>
<tr>
<th>Groups</th>
<th>MMP-2 (ng/mL)</th>
<th>TIMP-1 (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.67 ± 0.15</td>
<td>1893.85 ± 117.96</td>
</tr>
<tr>
<td>Carfilzomib (2 mg/kg)</td>
<td>6.08 ± 0.49</td>
<td>1533.85 ± 123.9</td>
</tr>
<tr>
<td>Carfilzomib (4 mg/kg)</td>
<td>6.31 ± 0.57</td>
<td>2012 ± 81.14</td>
</tr>
<tr>
<td>DENA 0.01% 10 weeks</td>
<td>11.5 ± 0.68a</td>
<td>6032.31 ± 335.9a</td>
</tr>
<tr>
<td>DENA + carfilzomib (2 mg/kg)</td>
<td>6.8 ± 0.46b</td>
<td>2387.86 ± 124.81b</td>
</tr>
<tr>
<td>DENA + carfilzomib (4 mg/kg)</td>
<td>6.56 ± 0.39b</td>
<td>2423.23 ± 138.38b</td>
</tr>
</tbody>
</table>

All data represent mean values ± SEM (n = 10).

Table 4. Effects of CFZ Treatment for 3 weeks on the Changes in Rat Hepatic Endostatin and Metallothionein Induced by DENA Intoxication

<table>
<thead>
<tr>
<th>Groups</th>
<th>Endostatin (ng/mL)</th>
<th>Metallothionein (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.06 ± 0.18</td>
<td>4.31 ± 0.34</td>
</tr>
<tr>
<td>Carfilzomib (2 mg/kg)</td>
<td>2.17 ± 0.1</td>
<td>5.23 ± 0.26</td>
</tr>
<tr>
<td>Carfilzomib (4 mg/kg)</td>
<td>2.24 ± 0.12</td>
<td>4.97 ± 0.31</td>
</tr>
<tr>
<td>DENA 0.01% 10 weeks</td>
<td>1.36 ± 0.03b</td>
<td>2.42 ± 0.12b</td>
</tr>
<tr>
<td>DENA + carfilzomib (2 mg/kg)</td>
<td>2.18 ± 0.11b</td>
<td>4.78 ± 0.21b</td>
</tr>
<tr>
<td>DENA + carfilzomib (4 mg/kg)</td>
<td>2.45 ± 0.17b</td>
<td>4.47 ± 0.33b</td>
</tr>
</tbody>
</table>

All data represent mean values ± SEM (n = 10).

Effects of CFZ Treatment on Hepatic MMP-2 Activity and TIMP-1 Activities

The treatment of normal rats with CFZ (2 and 4 mg/kg i.p) twice a week for 3 successive weeks from week 8 to week 10 did not alter hepatic MMP-2 and TIMP-1 activities as compared with their respective control groups (Table 3).

A two- and threefold increment in hepatic MMP-2 and TIMP-1 activities has been observed during induction of liver carcinogenesis as compared to their normal values (Table 3).

Three weeks treatment protocol with selected doses of CFZ provoked a remarkable inhibition in MMP-2 activity. MMP-2 activity has been markedly impeded reaching the normal values. Therefore, a treatment protocol with different doses of CFZ succeeded to normalize MMP-2 activity (Table 3). Moreover, 2 or 4 mg/kg i.p of CFZ treatment induced a similar profound inhibition in hepatic TIMP-1 (61 and 60%) (Table 3). However, the treatment protocol with different doses of CFZ did not succeed to normalize the TIMP-1 level and no tendency of the dose-related effect has been observed for the selected doses of CFZ on TIMP-1 (Table 3).

Effects of CFZ Treatment on the Changes of Hepatic Endostatin and MT Content

Administration of single dose of DENA (0.01%) in drinking water for 10 weeks induced a marked reduction in both hepatic endostatin and MT by about 34 and 44%, respectively, as compared to their respective normal values (Table 4). Treatment of rats with CFZ (2 or 4 mg/kg i.p) resulted in normalization in both hepatic endostatin and MT. Therefore, the treatment protocol with different doses of CFZ succeeded to normalize both endostatin and metallothionein contents (Table 4). The effect of CFZ treatment on endostatin tended to be dose related, while no tendency of the dose-related effect has been observed for CFZ on MT.

Histopathological Examination

Figure 1A demonstrated a normal liver histology with central hepatic vein. Moreover, liver sections taken
from normal rats treated with CFZ (2 and 4 mg/kg i.p) twice a week for 3 successive weeks showed normal liver histology with normal trabecular architecture of the hepatocytes (Figure 1B). The treatment of rats with DENA at a dose of 0.01% in drinking water for 10 weeks showed dysplastic hepatocytes with enlarged nuclei, coarse chromatin, and prominent nucleoli (Figure 1C). A group of animals that were given DENA at a dose of 0.01% in drinking water for 10 weeks and treated with CFZ (2 and 4 mg/kg i.p) twice a week for the last 3 successive weeks showed necrotic hepatocytes with nuclear karyolysis with many residual normal hepatocytes and very scanty residual mildly dysplastic hepatocytes in addition to normal hepatocytes showing bland nuclei and the absence of pleomorphism (Figure 1D).

**DISCUSSION**

In the present study, two representative tumor markers AFP and CEA are screened to explore their level variations with and without treatment. The data presented here demonstrate that the treatment of normal rats with DENA for 10 weeks in drinking water significantly increase serum tumor markers AFP and CEA four and two times, respectively. These results are...
in harmony with the previous results showing the significant rise in AFP and CEA levels obtained in animals treated with DENA [15–19]. Interestingly, treatment of rats with CFZ (2 and 4 mg/kg i.p) twice a week for the last 3 weeks with carcinogen intoxication prevents the rise in serum AFP and CEA levels. The observed significant inhibition in the levels of AFP and CEA in treated animals may suggest that CFZ can effectively alleviate the tumor burden in the rats.

VEGF is one of the first angiogenesis factors identified and the most important regulator of normal and tumor angiogenesis [20, 21]. Moreover, tumors depend on VEGF, TGF-β1, and FGF signaling for their growth and progression [22–24]. They stimulate angiogenesis and therefore can increase tumor vascularity, which can be inhibited by growth factors neutralizing antibodies [25–27]. Oral administration of DENA for 10 weeks induced 1.7, 3.2, and 2.5 increments in hepatic VEGF, FGF, and TGF-β1, respectively. It may be due to overproduction by the malignant lesions induced by DENA. The present data can confirm our previous report that VEGF and FGF elevated in early stage of multistep tumor development in liver initiated by DENA and promoted by CCl4 [19] and are supportive of the fact that VEGF and FGF promote the process of tumorigenesis [28, 29]. In the present study, the treatment protocol with the CFZ succeeded to induce a remarkably reduction in the highly elevated level of hepatic VEGF, FGF, and TGF-β1 and hence it may delay the process of hepatocarcinogenesis development induced by DENA.

MMPs play a crucial role in tumor cell invasion, metastasis, and angiogenesis [30, 31]. TIMP, such as TIMP-1 and TIMP-2, can regulate activities of MMPs in cancer cells [7]. Results of the present investigation revealed that chronic administration of DENA induced a marked elevation of MMP-2 activity associated with a highly significant increase in TIMP-1. The treatment protocol with CFZ afforded protection against hepatocarcinogenesis. The chosen doses of CFZ impeded hepatic MMP-2 activity and induced a pronounced inhibition of TIMP-1.

It has been suggested that transfer of human endostatin by an oncolytic adenovirus represents a potential approach for cancer therapy [32–34] because of its antiangiogenic effect. The present study demonstrates that DENA induced a profound inhibition in endostatin. The treatment protocol with the selected doses of CFZ (2 and 4 mg/kg i.p) normalized the hepatic endostatin level. Together with our previous data, we could clearly demonstrate that a proteasome inhibitor (CFZ) could attenuate the hepatic carcinogenesis development through preventing the decrease in the endostatin level [19].

The data presented here have clearly shown that DENA intoxication elicit a 44% reduction in the hepatic MT level. Our data stated that treatment with CFZ for the last 3 weeks along with carcinogen intoxication prevent the decrease in hepatic MT.

In conclusion, the present data declare that lower dose of CFZ may have a possible therapeutic potential against hepatocarcinogenesis and may afford a possible basis for uses of CFZ against human hepatic cancer.


