Possible role of selective, irreversible, proteasome inhibitor (carfilzomib) in the treatment of rat hepatocellular carcinoma

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Article info
Article history:
Received 28 November 2013
Received in revised form 25 February 2014
Accepted 5 March 2014
Available online 13 March 2014

Keywords:
Carfilzomib
Proteasome inhibitor
Hepatocellular carcinoma

A B S T R A C T
We investigated the possible therapeutic effect of irreversible proteasome inhibitor, carfilzomib against hepatocellular carcinoma induced chemically by chronic administration of diethylnitrosamines (DENA). Hepatocellular carcinoma induced by DENA in male Wistar rats was manifested biochemically by significant elevation of serum α-feto protein (AFP) and carcinoembryonic antigen (CEA). In addition, hepatic cancer was further confirmed by a significant increase in hepatic tissue growth factors; vascular endothelial growth factor (VEGF), transforming growth factor-β1 (TGF-β1) and basic fibroblast growth factor (FGF). Moreover a marked increase in matrix metalloproteinase-2 (MMP-2) and tissue inhibitor of metalloproteinase-1 (TIMP-1) content were also observed, along with a profound decrease in hepatic endostatin and metallothionein level.

Treatment of rats with the selected doses of carfilzomib produced a significant protection against hepatic cancer. The present results claimed that chosen doses of carfilzomib succeeded in suppressing serum tumor markers level AFP and CEA. Furthermore, the drug reduced the elevated level of hepatic growth factors, MMP-2 and TIMP-1 induced by the carcinogen. The antitumor effect of carfilzomib was also accompanied by augmentation of hepatic content of endostatin and metallothionein level. Histopathological examination of liver tissues also correlated with the biochemical observations. It could be concluded that treatment with carfilzomib confers a possible antitumor effect against hepatocellular carcinoma induced by DENA model in rats.

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1. Introduction

Human cancers treatment is limited by the unwanted adverse effect of chemostatic or chemotoxic anti-neoplastic agents. Hepatocellular carcinoma (HCC) is the most common type of liver cancer in the world with an estimated annual incidence of greater than 1 million new cases per year [1]. Up to now, there is no standardized, very effective approach for the treatment of inoperable HCC. Alternatively, several agents with more efficacies have been employed in treating HCC, however, its long term therapeutic outcome remains very poor [1]. The most commonly systemic chemotherapeutic agents are doxorubicin and 5-fluorouracil [2]. However these drugs are quite toxic and the results remain disappointing [2].

Changes in proteasome functions have been directly involved in the etiology of many cancers [3]. In general, specific malignancies can result from elevation of oncoproteins or breakdown of tumor suppressor genes products. Inhibition of proteasome functions has a potential antitumor activity [4].

Based on the great success achieved by application of bortezomib as a novel treatment for multiple myeloma (MM), a number of next-generation of proteasome inhibitors have been developed with the aims of improving efficacy, overcoming drug resistance, minimizing dose-limiting toxicity such as peripheral neuropathy (PN) and improving convenience of administration [5]. The recent accelerated approval of carfilzomib (tetrapeptide epoxyketone) is an example the success of this approach. The first study investigated the effect of proteasome inhibitors (PIs) in HCC was in 2004 demonstrating that MG-132 induced apoptosis in human HCC cells through caspase cascade leading to β-catenin cleavage and down-regulation of β-catenin-mediated trans-activation [6].
To investigate the possible antitumor effect of carfilzomib on liver cancer, we used the DENA for induction of rat hepatocellular carcinoma model. DENA is a well-known potent hepatocarcinogenic agent present in tobacco smoke, water, cured and fried meals, cheddar cheese, agricultural chemicals, cosmetics and pharmaceutical products [7–9]. DENA is known to induce damage in many enzymes involved in DNA repair and is normally used to induce liver cancer in experimental animal models [10]. After its use, many studies have investigated a series of microscopic lesions called "foci" and "nodules" which have been designated "preneoplastic" or "premalignant" [11]. During the process of neoplastic transformation, various histochemical and biochemical marker enzymes and protein antigens are expressed depending upon the stages and magnitude of neoplasia. These markers are frequently considered as surrogate end-point biomarkers in rat liver carcinogenesis model [12].

To date and up to our knowledge, there are no published studies investigating the possible effect of irreversible proteasome inhibitor; carfilzomib against hepatic cancer induced by DENA. Therefore, the present work was undertaken to investigate the possible antitumor effect of different doses of carfilzomib. Moreover, to identify the underlying mechanisms by studying the effect of selected doses of the drug on the different hepatic growth factors, MMP-2, TIMP-1, endostatin, and metallothionein as an index of antioxidant status.

2. Materials and methods

2.1. Animals

Adult male albino rats of the Wistar strain (170–200 g) 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 diet pellet and fed chow spruce and water ad libitum. All animal procedures followed the international guidelines of proper experimental animal handling.

2.2. Chemicals

Diethylnitrosoamine will be obtained from Santa Cruz Biotechnology, Inc. 2145 Delaware Avenue Santa Cruz, CA. 95060 USA. Carfilzomib was obtained from Active Biochem. Redan, GA 30074 USA, 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, Inc. USA. Endostatin, metallothionein, carcinoembryonic antigen (CEA), alpha-fetoprotein (AFP) were obtained from Uscnlife Science & Technology CO. LTD (Wuhan, China) according to manufacturer's instructions.

2.3. Experimental protocol

The animals were divided randomly into 6 groups, 10 animals in each group. The first group (control) received vehicles used for carfilzomib, DMSO (0.4 ml/kg i.p.) twice a week from week 16 to week 18. The second and third groups received carfilzomib (2 and 4 mg/kg i.p.) twice a week from week 16 to week 18, respectively [13]. The fourth group was given DENA at a dose of 0.01% in drinking water for 15 weeks, 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 16 to week 18 [14,15]. The last two groups were given DENA at a dose of 0.01% in drinking water for 15 weeks [13] and treated with carfilzomib (2 and 4 mg/kg i.p.) twice a week from week 16 to week 18, respectively [13]. 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 non-heparinized tubes. Serum was separated by centrifugation for 5 min at 1000g 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, Conn., USA).

2.4. Assessment of biochemical parameters

2.4.1. Enzyme linked immunosorbent assay (ELISA) of AFP and CEA

Quantitative estimation of hepatic tumor markers AFP and CEA were based on ELISA using assay kits from Usclnte Science & Technology Co. LTD (Wuhan, China) according to manufacturer's instructions.

2.4.2. Enzyme linked immunosorbent assay (ELISA) of different growth factors, matrix metalloproteinase-2, tissue inhibitor of metalloproteinase-1, endostatin, and metallothionein levels in liver homogenates

VEGF, TGF-β1, FGF, MMP-2 and TIMP-1 were assayed in the liver homogenates by ELISA using assay kits from R&D systems (Minneapolis, MN) according to manufacturer's instructions. In addition, endostatin, metallothionein were also analyzed by ELISA using assay kits from Usclnte Science & Technology Co. Ltd (Wuhan, China) according to manufacturer's instructions.

2.5. Histopathological evaluation

Histopathological evaluation was performed on the liver and a portion of the specimen was fixed in 10% formalin and embedded in paraffin wax. Sections were cut at 4 μm thicknesses, stained with hematoxylin and eosin and viewed under light microscope. To avoid any type of bias, the slides were coded and examined by two histopathology's who were blinded to the treatment groups. It was investigated for the presence of hepatic cirrhosis, hepatocellular dysplasia (dysplastic cirrhotic nodules) and frank hepatocellular carcinoma formations. The size of malignant hepatic foci in six rats from DENA-intoxicated and carfilzomib treated hepatic cancer induced by DENA intoxication groups were measured using a microscopic eye piece graticule. Measurements were done on three sections taken from each animal liver. A high power Nikon Eclipse 80i objective (40×) was used and measurements were done using the eye piece graticule on 0.59 mm field diameter space.

2.6. Statistical analysis

Data are expressed as means ± SEM (n = 10). Statistical comparison between different groups were done using one way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparison tests, to judge the difference between various groups. Significance was accepted at P < 0.05.
3. Results

3.1. Effect of carfilzomib treatment on the elevated serum hepatic tumor markers levels induced by carcinogen intoxication

Treatment of normal rats with different doses of carfilzomib (2 and 4 mg/kg i.p.) twice a week for 3 weeks 16–18 did not significantly affect the measured serum hepatic tumor markers as compared with their control groups (Fig. 1A, B). However, oral administration of DENA (0.01%) in drinking water for 15 weeks induced a significant 10- and 4-fold increments in both serum hepatic tumor markers; AFP and CEA, respectively as compared with their control groups.

Treatment of hepatic cancer induced by carcinogen intoxication for 15 weeks with the chosen doses of carfilzomib (2 and 4 mg/kg i.p.) twice a week for the last 3 weeks 16–18, resulted in 44.6% and 50.5% decrease in serum AFP, respectively (Fig. 1A) and 49.9% and 46.39% reduction in serum CEA, respectively (Fig. 1B) as compared with DENA group. However, the serum level of hepatic tumor markers still significant higher than the control groups. No tendency of dose-related effect has been observed for carfilzomib on different hepatic tumor markers levels.

3.2. Effects of carfilzomib administration on hepatic growth factors; VEGF, TGF-β1 and FGF

Treatment of normal rats with carfilzomib (2 and 4 mg/kg i.p.) twice a week for 3 weeks 16–18 showed a non-significant changes in hepatic growth factors VEGF, TGF-β1 and FGF as compared with their respective control groups (Fig. 2A–C).

Long term administration of selected dose of DENA 0.01% in drinking water induced 2.4-, 5.1- and 5.2-fold increase in hepatic VEGF, TGF-β1and FGF, respectively as compared with their corresponding control groups. Administration of selected doses of carfilzomib (2 and 4 mg/kg i.p.) twice a week for the last 3 weeks induced a profound reduction of hepatic VEGF level 39.24%, 37.31%, respectively (Fig. 2A) as compared with DENA group. Moreover, a similar pattern of significant reduction in the hepatic TGF-β1 65.15% and 63.55% (Fig. 2B) and FGF 35.77% and 27.44% (Fig. 2C) were also observed as a result of administration of aforementioned doses of carfilzomib. However, No tendency of dose-related effect has been observed for carfilzomib on different growth factors levels. The hepatic level of growth factors remains significant higher than their respective control values.
3.3. Effects of carfilzomib treatment on hepatic MMP-2 activity and TIMP-1

Treatment of normal rats with carfilzomib (2 and 4 mg/kg i.p.) twice a week for 3 successive weeks 16–18 did not induce any significant changes in hepatic MMP-2 and TIMP-1 as compared with their respective control groups (Fig. 3A, B).

A threefold increment in hepatic MMP-2 activity has been observed during induction of liver cancer as compared to their normal value (Fig. 3A). Similarly, a fivefold increments in hepatic TIMP-1 content have been observed during carcinogen administration as compared to normal value (Fig. 3B).

Three weeks treatment protocol with selected doses of carfilzomib provoked a pronounced inhibition in MMP-2 activity. Therefore, MMP-2 activity has been markedly impeded (36.9% and 42.4%) (Fig. 3A). The effect of carfilzomib treatment on MMP-2 tended to be dose related. A similar remarkable inhibition (47.6% and 44.5%) in hepatic TIMP-1 induced by 2 or 4 mg/kg i.p. of carfilzomib treatment (Fig. 3B). However, treatment protocol with different doses of carfilzomib did not succeed to normalize MMP-2 activity and TIMP-1 level.

3.4. Effects of carfilzomib treatment on the changes of hepatic endostatin and metallothionein content

Administration of single dose of DENA (0.01%) in drinking water for 15 weeks induced a similar marked reduction in both hepatic endostatin and metallothionein by about 64.6% and 66.25%, respectively as compared to their respective normal values (Fig. 4A, B). Treatment of rats with 2 or 4 mg/kg i.p. of carfilzomib resulted in 83.7% and 90% enhancement in hepatic endostatin, respectively (Fig. 4A) and 76.6% and 69.3% elevation of hepatic metallothionein, respectively (Fig. 4B). However, elevated level of hepatic endostatin and metallothionein did not reach their normal values. The effect of carfilzomib treatment on endostatin tended to be dose related, while no tendency of dose related effect has been observed for carfilzomib on metallothionein.

3.5. Histopathological observation

Fig. 5A showed normal hepatocytes adjacent to a portal tract within which a vein and bile ducts were identified. Moreover liver sections taken from normal rats treated with carfilzomib (2 or 4 mg/kg i.p.) twice a week for the last 3 successive weeks showed normal liver histology with preserved normal hepatic trabecular architecture (Fig. 5B, C).

Treatment of rats with DENA at a dose of 0.01% in drinking water for 15 weeks showed cirrhotic liver nodules of variable sizes surrounded by proliferated and dilated bile duct and showing early stages of cholangiocarcinoma, malignant hepatocytes with large nuclei and prominent nucleoli, abnormal mitosis and well differentiated hepatocellular carcinoma formation in addition to complete loss of normal “one cell thick” trabecular architecture pattern of the liver were observed (Fig. 5D).

A group of animals who were given DENA at a dose of 0.01% in drinking water for 15 weeks and were treated with carfilzomib (2
Fig. 5. Photomicrographs of liver specimens stained with H&E. Histopathological observation. (A) Showed normal hepatocytes adjacent to a portal tract within which a vein and bile ducts were identified. Moreover, liver sections taken from normal rats treated with carfilzomib (2 and 4 mg/kg i.p.) twice a week for 3 successive weeks showed normal liver histology with preserved normal hepatic trabecular architecture (B, C). Treatment of rats with DENA at a dose of 0.01% in drinking water for 15 weeks showed cirrhotic liver nodules of variable sizes surrounded by proliferated and dilated bile duct and showing early stages of cholangiocarcinoma, malignant hepatocytes with large nuclei and prominent nuclei, abnormal mitosis and well differentiated hepatocellular carcinoma formation in addition to complete loss of normal “one cell thick” trabecular architecture pattern of the liver were observed (D). A group of animals that were given DENA at a dose of 0.01% in drinking water for 15 weeks and were treated with carfilzomib (2 and 4 mg/kg i.p.) twice a week for another 3 successive weeks after 15 weeks showed apoptosis and cellular necrosis within nodules of liver carcinoma (E, F). (G) Effect carfilzomib (2 mg/kg i.p.) treatment twice a week for 3 weeks on the hepatic foci induced by DENA intoxication. Carfilzomib (CFZ) 2 mg/kg i.p. was administered twice a week for 3 weeks. The results were expressed as malignant foci (cm). Each column represents the mean of 10 rats with a vertical bar showing SEM. *Significant difference from DENA group at P < 0.05.
or 4 mg/kg i.p.) twice a week for another 3 successive weeks showed apoptosis and cellular necrosis within nodules of liver carcinoma (Fig. 5E, F). Oral administration of DENA (0.01%) in drinking water for 15 weeks induced significant malignant foci in hepatic tissues. Fig. 5G showed that the size of malignant foci ranged from 0.6 to 1.2 cm (0.9 ± 0.11) (mean ± SEM). Treatment of rats with carfilzomib 2 mg/kg i.p. induced a significant decrease in the size of hepatic foci (p < 0.05). The size of malignant foci ranged from 0.3–0.6 (0.466 ± 0.05) (mean ± SEM).

4. Discussion

The present study demonstrates a possible role of irreversible proteasome inhibitor; carfilzomib in treatment of hepatocellular carcinoma induced chemically by long term administration of DENA. Three weeks administration of the chosen doses of carfilzomib suggested a possible role of carfilzomib in the treatment of hepatic cancer.

Tumor markers are widely applied to evaluate tumor diagnosis, treatment and prognosis. Relevant studies revealed that the levels of tumor markers can be changed before and after treatment, whereas its specific mechanism still remains undefined. Meanwhile, some studies demonstrated that it may be related to decreased tumor burden [16–18].

Among tumor markers, AFP and CEA are screened to explore their level variations with and without treatment and may suggest proteasome inhibitors of carfilzomib against hepatic cancer. Therefore, the effect of carfilzomib treatment protocol on VEGF, FGF and TGF-β1 are screened to explore the mechanism of action of proteasome inhibitor carfilzomib against hepatic cancer. Therefore, the effect of carfilzomib treatment protocol on VEGF, FGF and TGF-β1comes into light for the first time in this study.

Increasing attention in recent years. Vascular endothelial growth factor (VEGF) is one of the first angiogenesis factors identified. It is the most important regulator of normal and tumor angiogenesis [24,25]. Moreover, tumors depend on both VEGF and FGF signaling for their growth and progression [26–28]. The extensive cross-talk between VEGF and FGF pathways plays a role in promoting angiogenesis and tumor growth, as well as in mechanisms of anti-angiogenic therapy escape. FGF induces angiogenesis through different oncogenic pathways, including VEGF, resulting in increased tumor growth and metastases [29]. FGF and VEGF have been found to act synergistically, with their co-expression being associated with more aggressive tumor growth in xenografts [30]. Huang et al. [31] have demonstrated that expression of fibroblast growth factor receptor1 (FGFR1) in the liver accelerated hepato-carcinogenesis initiated by DENA in transgenic mice. In cooperation with initiators as DENA, FGFR1 is a strong promoter of liver cancer through promotion of rate of cell cycling and neo-angiogenesis.

Likewise, transforming growth factor-β1 (TGF-β1) is multifunctional polypeptide that can influence tumor cell behavior by directly binding to TGF-β1 receptors located on the tumor cells themselves or by influencing the peri-tumoral milieu. It stimulates angiogenesis and therefore can increase tumor vascularity, which can be inhibited by TGF-β1 neutralizing antibodies [32–34]. These growth factors are the most sensitive and dramatic indicators of cancer especially hepatocellular carcinoma, as they are remarkably increased when hepatic cells give rise to malignant transformation after carcinogen intoxication [25]. In the present study, three growth factors VEGF, FGF and TGF-β are screened to explore the mechanism of action of proteasome inhibitors of carfilzomib against hepatic cancer. Therefore, carfilzomib suggested a possible role of carfilzomib in the treatment of hepatic cancer.

Endostatin specifically acts on neovascular endothelial cells, inhibits cell migration, and induces cell apoptosis, thus playing a major antiangiogenic role by acting on tumor-associated neovascular endothelial cells [38,39] and has been shown to elicit antitumor effect in many solid tumors. Fang et al. [40] suggested that transfer of human endostatin by an oncolytic adenovirus represents a potent approach for cancer therapy. The present study demonstrates that DENA induced a profound inhibition in endostatin level. Treatment with the selected doses of carfilzomib (2 and 4 mg/kg i.p.) twice a week for 3 weeks induced 46% and 48% increment in endostatin level. Together with our previous data [35], we could clearly demonstrate that carfinogen intoxication induced a profound inhibition in hepatic endostatin level in pre-malignant and malignant lesions and may suggest proteasome inhibitor; carfilzomib could attenuate the hepatic carcinogenesis development through prevention the decrease in endostatin level [35].
The data presented here have clearly shown that, DENA intoxicated rats elicited a 66% reduction in hepatic metallothionein level. Metallothioneins (MT), a group of stress response proteins induced at a high level by oxidative stress, are efficient scavengers of reactive oxygen species (ROS) and reactive nitrogen species. The previous studies showed that the relative lack of MT expression in pre-neoplasia was consistent with similar observations on hepatozellular, colorectal and papillary thyroid carcinomas [41–43]. Moreover, histochemical analysis has shown that MT level correlates inversely with tumor grade in hepatocarcinogenesis [44,45]. Therefore, depletion of hepatic MT in malignant tumor developed in rats up on DENA administration suggests a protective role for MTs against chemical carcinogens. Our data stated that, treatment of hepatic cancer with carfilzomib for 3 weeks induced a significant elevation of hepatic MT level. These data may reflect the protective effect achieved by chosen doses of carfilzomib against hepatic cancer development initiated by DENA. These data are compatible with our previous data [35].

Matrix metalloproteinases (MMPs) are a class of zinc-dependent endopeptidase enzymes, that play a crucial role in extracellular matrix (ECM) components degradation and tumor cell invasion, metastasis and angiogenesis [22,27]. MMP-2 and MMP-9 degrade most of the ECM components of basal membrane and type IV collagen, a major component of the basement membrane. Activities of MMPs are controlled by their endogenous inhibitors, metalloproteinases (TIMPs) such as TIMP-1 and TIMP-2, in cancer cells [8]. It was reported that when the balance of MMPs and TIMPs was broken, direct inhibition of MMPs and increase of TIMPs in cancer may be a particularly attractive target for therapeutic intervention in tumor invasion and metastasis [46].

In an attempt to further elaborate the mechanism of action of carfilzomib in hepatocellular carcinoma, we studied their effects on MMP-2 and TIMP-1 in hepatic tissue. 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. Treatment protocol with two doses carfilzomib afforded protection against hepatic cancer. The chosen doses of carfilzomib induced a pronounced inhibition of MMP-2 and TIMP-1. However, their hepatic activities remain significant higher than their control groups. No tendency of dose-related effect has been observed for carfilzomib on MMP-2 and TIMP-1 hepatic levels.

In conclusion: the present data strengthen the argument that lower dose of carfilzomib (2 mg/kg) can be used in therapy of hepatic cancer. This action could probably be mediated via inhibition of growth factors-mediated angiogenesis and enhancement of endostatin mediated anti-angiogenesis, increase in metallothionein, reduction of MMP-2 and TIMP-1 in addition to induction of apoptosis in transformed cells. Therefore, lower dose of carfilzomib may have a possible therapeutic potential in the treatment of hepatic cancer. Thus, the present study could afford a possible basis for uses of carfilzomib against human hepatic cancer.

Conflict of interest

There are no financial or other interests with regard to this manuscript that might be construed as a conflict of interest.

Acknowledgements

This work was supported by King Abdul-Aziz City for Science and Technology (KAUST: AT-34-25) and Research Center, College of Pharmacy, King Saud University and College of Graduate Studies, King Saud University.
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