PRECLINICAL STUDIES

# Ent-11α-hydroxy-15-oxo-kaur-16-en-19-oic-acid inhibits hepatocellular carcinoma *in vitro* and *in vivo* via stabilizing IkBα

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**Summary** Ent-11-hydroxy-15-oxo-kaur-16-en-19-oic-acid (5F) isolated from *Pteris Semipinnata* L is known to inhibit certain tumor cells *in vitro*. The information on the *in vivo* effect of 5F is limited and its effect on hepatocellular carcinoma (HCC) is unknown. In this study, the anti-tumor effect of 5F was investigated in a diethylnitrosamine (DEN)-induced mouse HCC model. In addition to therapeutic effect, the potential side effect was monitored. A panel of cultured HCC cells was used to confirm the *in vivo* data and explore the responsible molecular pathway. The result showed that 5F significantly inhibited the DEN-induced HCC tumors by reducing the number of tumor foci and the volume of tumors. Furthermore, 5F induced the death of cultured HCC cells in dose- and time-dependent manners. The cell death was confirmed to be apoptotic by *in vivo* and *in vitro* TUNEL assays. 5F inhibited NF-kB by stabilizing its inhibitor IkBα, reducing the nuclear p65 and inhibiting NF-kB activity. Subsequently it affected the NF-kB downstream molecules with a decrease in anti-apoptotic Bcl-2 and increase in pro-apoptotic Bax and Bak. During the whole period of the experiment, mice receiving 5F appeared to be healthy, though they suffered from a mild degree of hair loss. 5F did not damage liver and renal functions. In conclusion, 5F is effective against HCC with minimal side effects. It induces apoptosis in HCC cells via inhibiting NF-kB, leading to the decrease of Bcl-2 but the increase of Bax and Bak.

**Keywords** Hepatocellular carcinoma ·
Ent-11-hydroxy-15-oxo-kaur-16-en-19-oic-acid ·
Apoptosis · Toxicity · Nuclear factor-kappaB

## Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers affecting millions of individuals worldwide. Surgical treatments of HCC, including resection, transplantation, and local ablation, are considered as a potentially curative therapy. However, about 80–90% of patients are not eligible for such treatments at diagnosis [1]. The treatment options for these patients are mainly palliative, such as transarterial chemoembolization, intra-arterial or systemic chemotherapy, radiotherapy and immunotherapy, and the 5-year relative survival rate for them is only 7% [2]. Moreover, patients who have received surgical treatments are also at risk of recurrence at a rate of approximately 50% at 3 years [2]. The above situations highlight the urgent need for new therapeutic approaches for this malignancy.

Ent-11α-hydroxy-15-oxo-kaur-16-en-19-oic-acid is the fifth fraction isolated from *Pteris Semipinnata* L. For simplicity, this compound is termed as 5F. 5F is able to kill

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various human cancer cells including thyroid cells, nasopharyngeal carcinoma cells, gastric cancer cells, and colorectal cancer cells *in vitro* [3–9]. Experiments have shown that 5F induces apoptosis of human cancer cells in a concentration- and time-dependent manner [6–8]. Studies have suggesting that cell death induced by 5F is through a mitochondrial-mediated pathway. However, the information of *in vivo* anti-tumor effect of 5F is limited, and its potential side-effect is basically unknown. The anti-HCC property of 5F has also not yet been investigated. This study therefore was to determine the tumorcidal effect of 5F on *in vitro* cultured HCC cells and an *in vivo* HCC mouse model.

## Materials and methods

*Chemicals and reagents* 5F was prepared as the previous description [3, 5]. All antibodies used were obtained from Santa Cruz (Santa Cruz, CA). Other reagents, if not specifically indicated, were purchased from Sigma (St Louis, MO).

*Animal model and treatment, and tumor calculation* Infant C3H/HeJ mice (Bar Harbor, ME) at 4–6 days old were randomly divided into 8 groups detailed in Table 1 and each group contained 10 mice. The liver carcinogen diethylnitrosamine (DEN) was used to induce HCC in the mice according to the published procedure [10, 11]. After the administration of DEN, the tumor was allowed to develop for 17 weeks. 5F treatment started at the first day of the 17th week. 5F dissolved in saline was intraperitoneally injected at designated concentrations (Table 1), and given for 5 consecutive days per week. Three types of controls were set up. Mice received DEN only were the positive tumor control (Group 4). Mice without DEN induction but treated with 5F were the 5F-only control (Groups 5–7). Mice received saline only were the vehicle control (Group 8). The

treatment lasted for 12 weeks. The mice were maintained under standardized conditions in the Research Animal Resources facility of our institute according to the ethical animal guideline. The weight of individual mouse was monitored bi-weekly. The blood was drawn by direct venal puncture at the posterior vena cava. Livers of all mice were dissected and weighted after the completion of treatments. Four sections taken from the different parts of the liver were used for hematoxylin and eosin (H&E) staining. The number of tumors was counted under the light microscopy. The volume of each visible tumor was calculated by a formula: volume=$\pi d/2$ [12, 13], where d = diameter of the tumor, and the total volume of the tumors in each individual mouse was accordingly determined.

*Biochemical parameters related to liver and renal functions* Mouse blood was collected and serum was isolated for testing aspartate aminotransferrase (AST), alanine aminotransferrase (ALT), serum creatinine (CRE), and blood urea nitrogen (BUN) to determine the side-effect of 5F. The former two parameters were used to test the liver function, and the latter two the renal function. The assay kits for the above 4 parameters were provided by King Hawk Pharmaceutics (Beijing, China) and performed according to the manufacturer's instructions. The reference ranges for these parameters in mice are: AST<37U/L, ALT<40U/L, CRE< 106 µmol/L, BUN range of 1.8–7.1 mmol/L and panic concentration >35.7 mmol/L [14].

*Immunohistochemistry* Immunohistochemical staining of proliferating cell nuclear antigen (PCNA), Bcl-2, Bcl-xL, Bax, Bak, Bid, p65, and I-kBα was performed and the positive staining was graded as described previously [6, 15]. Briefly, samples with less than 10% of positive cells in 20 random high-power fields was scored as 1; samples with 10%–30% of positive cells were positive as 2; samples with 30%–50% of positive cells were positive as 3 and samples with more than 50% of positive cells were positive as 4.

*Cell cultures* The human liver cell lines SK-Hep-1, Huh-7, PLC/PRF/5, HepG2 and Hep3B were cultured as described previously [16, 17].

*Cell viability and apoptosis* Cell viability was measured by MTT assay [16, 17]. Apoptosis of cultured cells was determined by APO-BRDU Flow cytometry kit for apoptosis (Phoenix Flow Systems, San Diego, CA) [3, 18]. The *in vivo* apoptotic cells was assessed by *In Situ* Cell Death Detection Kit (TUNEL assay) (Roche, Penzberg, Germany) and performed according to the instructions of the manufacturers.

*Western blot* Western blot was carried out as described previously [6, 9].

**Table 1** Mouse groups, treatments and hair loss

| Group | Treatment | Hair loss scale |
|---|---|---|
| 1 | DEN+5F 12 mg/kg | + |
| 2 | DEN+5F 24 mg/kg | + |
| 3 | DEN+5F 48 mg/kg | ++ |
| 4 | DEN only | 0 |
| 5 | Saline+5F 12 mg/kg | + |
| 6 | Saline+5F 24 mg/kg | + |
| 7 | Saline+5F 48 mg/kg | + |
| 8 | Saline only | 0 |

0: No visible hair loss; +: Hair loss observed after handling; ++: Noticeable hair loss, localized area; +++: Majority skin surface visible; ++++: Complete hair loss (nude)

*NF-kB p65 activity* The nuclear extract was isolated for the measurement of p65 activity, which was determined with an NF-κB (p65) Transcription Factor Assay ELISA kit from Cayman Chemical (Ann Arbor, MI) and performed according to the manufacturer's instruction.

*Statistical analysis* Data were expressed as mean±SD. Statistical differences between groups were examined using the One-Way ANOVA and student's *t* test. There was statistically significant difference when $P < 0.05$.

# Results

## Effects of 5F on the number and volume of tumors

The *in vivo* effect of 5F was examined in a DEN-induced mouse HCC model and three different doses of 5F were given (Table 1). It was found that 5F at 24 and 48 mg/kg could significantly reduce the number of tumors induced by DEN, compared with mice without 5F treatment (Fig. 1a, b). Similar effects were observed on the volume of tumors. The lowest dose of 5F (12 mg/kg) also decreased the volume of tumors compared with mice received DEN only (Group 4). However, the reduction did not reach a significant point. The *in vivo* tumorcidal effect of 5F was dose-dependent (Fig. 1a, b).

## Potential side-effects of 5F

5F treatment did not significantly affect the body weight of mice (Fig. 1c). It was found that mice treated with 5F had some hair loss compared with mice treated with DEN or saline only, however, this effect was not serious (Table 1). During the whole period of the experiment, no mice showed any sign of intolerance to 5F, decrement of activity, self-isolation, self-torture, and other visible abnormalities.

In order to assess the possible toxic effects of 5F on liver and kidney, we measured 4 biochemical parameters that can reflect the liver and renal functions *in vovo*. In the liver function test, the levels of AST and ALT were within the normal reference range in all groups tested (Fig. 1d). This indicates that 5F treatment did not result in liver dysfunction in the model tested. In the renal function test, CRE concentrations of all groups were normal (Fig. 1e). The level of BUN was slightly increased in all mice tested (Fig. 1e). It was apparent that the increased value of BUN was still much lower than the panic level [14]. Furthermore, there was not difference between 5F-treated mice and untreated ones, and all mice appeared to be normal during the course of the experiment. Therefore, the results of biochemical parameters tested and physiological observations indicate that 5F treatment did not result in major side-effects.

## Inhibitory effect of 5F on the cultured HCC cells

The inhibitory effect of 5F was tested in 5 HCC cell lines, SK-Hep-1, Huh-7, PLC/PRF/5, HepG2, and Hep3B. 5F significantly reduced cell viability in all cell lines tested (Fig. 2a). SK-Hep-1 cells were the most sensitive to 5F whereas Hep3B cells were the least sensitive. The order of the sensitivity to 5F treatment was SK-Hep-1>Huh-7>PCL/PFR/5>HepG2>Hep3B. It appeared that the inhibitory effect of 5F was dose- and time-dependent.

To determine whether cell death caused was apoptotic, DNA fragmentation of HCC cells treated with 5F was assessed by TUNEL. The result showed that 5F increased DNA fragmentation of PLC/PRF/5 cells in a concentration-dependent manner (Fig. 2b). The similar result was obtained in the other 4 cell lines (data not shown). *In situ* TUNEL assay also revealed that the TUNEL positive cells were much higher in HCC cells of mice treated with 5F than HCC cells of mice without 5F treatment or liver cells of non-tumor bearing mice treated with 5F or saline (Fig. 2c).

## Proliferative and apoptotic molecules changed by 5F

We examined three groups of molecules that are associated with cell proliferation and apoptosis in the DEN-induced HCC mice receiving 5F and cultured HCC cells treated with 5F. The first molecule was PCNA, a cell proliferation marker [19]. The level of PCNA was much higher in HCC liver of DEN-induced mice (Group 4) compared with all other groups (Fig. 3). The reduced expression of PCNA appeared to be dose-dependent in HCC mice treated with 5F. Thus, the finding of PCNA supports the data of cell death and apoptosis in HCC cells treated with 5F (Fig. 2). The second group was Bcl-2 family members: Bcl-2, Bcl-xL, Bax, Bak and Bid. These molecules have been reported to be affected by 5F in other tumor cells [3, 6, 9]. We found that the level of Bcl-2 was increased in HCC liver of DEN-induced mice (Group 4) and 5F treatment significantly decreased its level (Fig. 3). In contrast to Bcl-2, the levels of Bax and Bak were enhanced by 5F treatment (Fig. 3). However, we did not find any difference in the expression of Bcl-xL and Bid between mice treated with 5F and those without 5F treatment (data not shown). The third group tested was nuclear factor kappaB (NF-kB) family, p65, a subunit of NF-kB, and IkBα, an inhibitor of NF-kB [20]. It was found the level of p65 was inhibited by 5F whereas the level of IkBα was up-regulated by 5F (Fig. 3). The changes in both molecules appeared to be in a dose-dependent manner. The alterations of these cell proliferative and apoptotic molecules by 5F *in vivo* were supported by the result obtained in *in vitro* cultured HCC cells treated with 5F (Fig. 4a).

The role of NF-kB in 5F-induced cell death was further explored and verified by different approaches. First, 5F
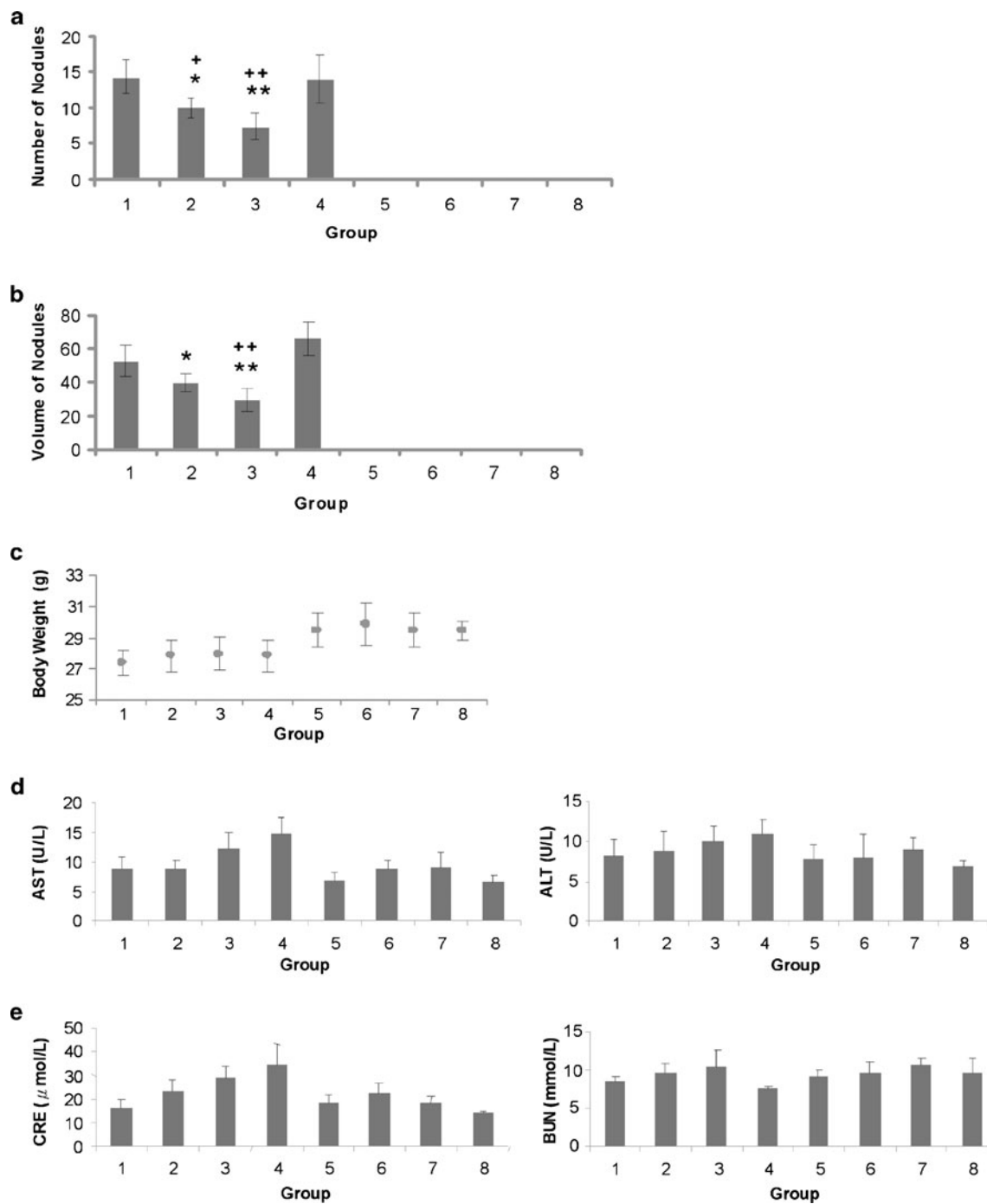
Fig. 1 Tumorcidal effect of 5F on mouse HCC and potential side-effects. C3H/HeJ mice were randomly divided into 8 groups (Table 1). Each group of mice contains 10 mice. HCC was induced by diethylnitrosamine (DEN). After 16 weeks of DEN induction, 5F was given via i.p for 5 consecutive days per week. Saline was administered via i.p. as vehicle control (Group 8). 5F treatment lasted for 12 weeks. At the end of treatment, the number of liver tumors **a** and the volume of total tumors **b** in each liver were determined according to the procedure described in the Materials and methods. *$P<0.05$, compared with mice treated with DEN only (Group 4); **$P<0.01$, compared with mice treated with DEN only; +$P<0.05$, compared with mice treated with 12 mg/kg 5F (Group 1); ++$P<0.01$, compared with mice treated with 12 mg/kg 5F. The potential side-effects of 5F were determined by body weight, liver and renal functions. The body weight of individual mouse was measured bi-weekly and the body weight taken immediately before the end of the experiment was shown **c**. The liver function was reflected by measuring serum AST and ALT **d** and the renal function by measuring serum CRE and BUN **e**. At the end of mouse experiment, blood was taken and serum was separated for the measurement of AST, ALT, CRE and BUN using relevant assay kits

**Fig. 2** Effect of 5F on the death of HCC cells. **a** HCC Cells were treated with 5F (0–150 mg/L) for 24 h, 48 h, and 72 h. Cell viability was measured by MTT assay and the data were expressed as a percentage of the control culture conditions (0 mg/L 5F). The data were represented as the mean±SD for four replicate determinations for each treatment. Significant cell death in all types of HCC cells was induced by 5F as p values showed at each cell line tested. **b** Cultured HCC cells were treated with various concentrations of 5F for 48 h. At the end of treatment, apoptotic cells were determined by APO-BRDU Flow cytometry kit for apoptosis and the percentage of apoptotic cells was calculated. 5F significantly induced apoptosis in all HCC cells tested (all $P<0.01$, ANOVA). **c** Liver tissue samples were collected from each group of mice (Table 1) at the end of the experiment and apoptosis was determined by *In Situ* Cell Death Detection Kit (TUNEL assay). *$P<0.05$, compared with mice treated with 48 mg/kg 5F (Group 3); ++$P<0.01$, compared with mice treated with DEN only (Group 4); ^^$P<0.01$, compared with 5F-treated mice at the corresponding doses (Groups 1–3) and ##$P<0.01$, compared with mice treated with saline only (Group 8)
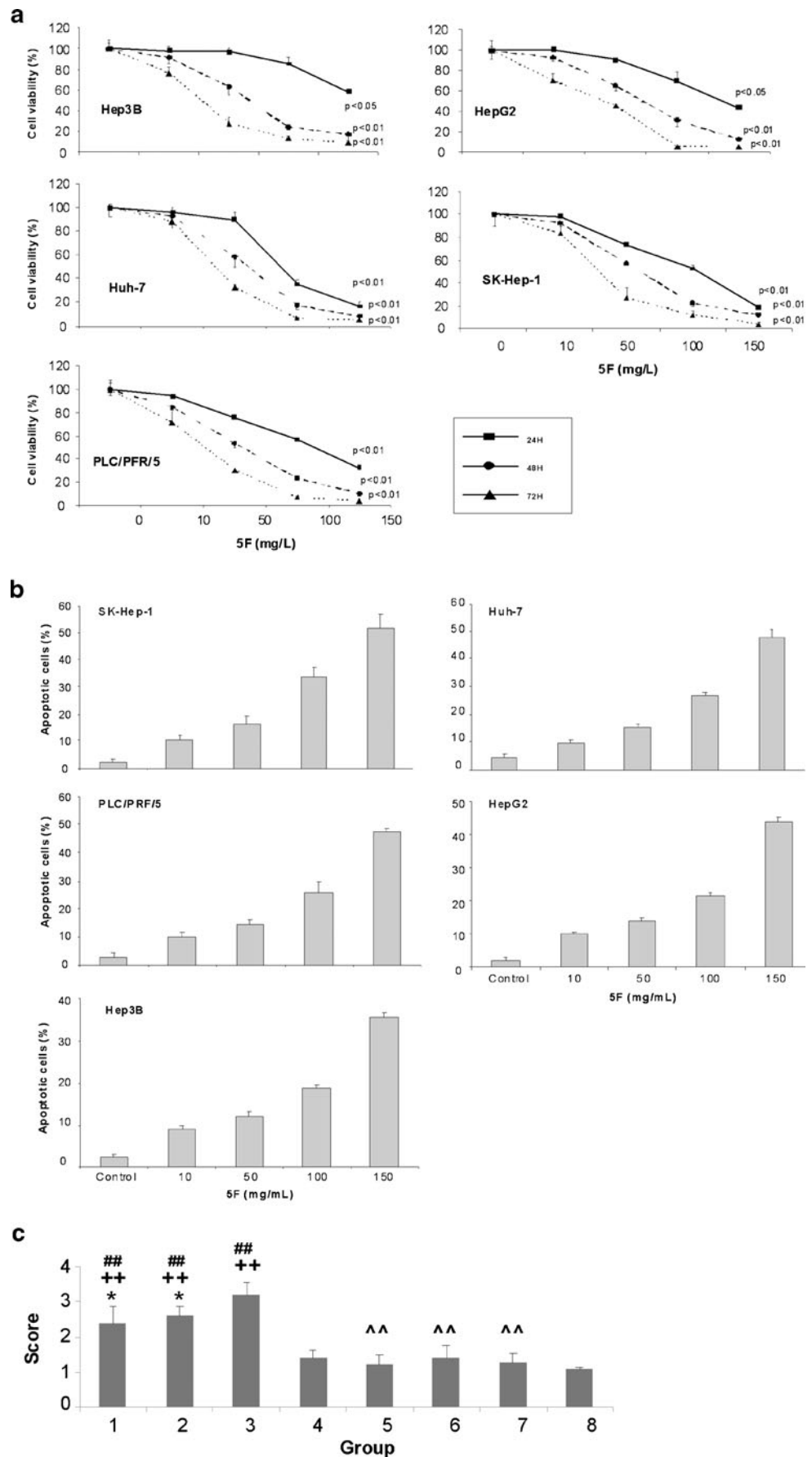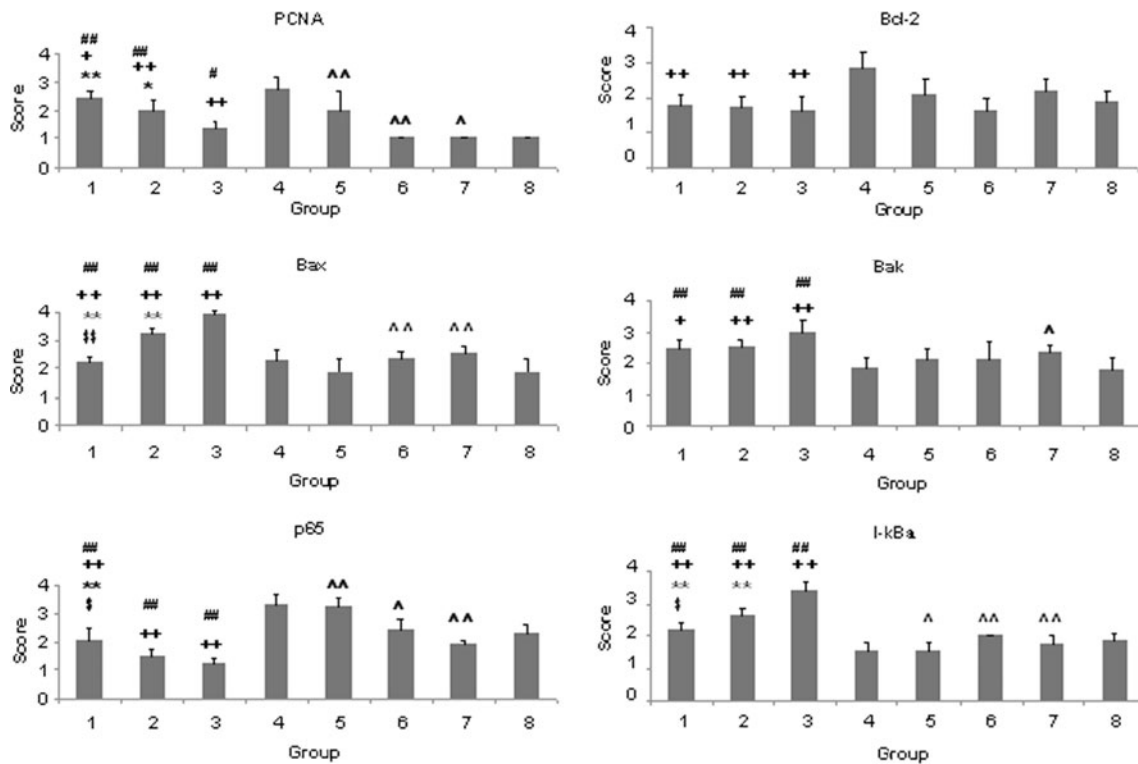
**Fig. 3** Effect of 5F on protein expression in the HCC mouse model. Liver tissue samples were obtained from each group of mice (Table 1) at the end of the experiment. The expression of the relevant protein marker was measured by immunohistochemical staining. The positive staining was scored as described in the Materials and Methods. *$P<0.05$, compared with mice treated with 48 mg/kg 5F (Group 3); **$P<0.01$, compared with mice treated with 48 mg/kg 5F (Group 3); \$$P<0.05$, compared with mice treated with 24 mg/kg 5F (Group 2); \$\$$P<0.01$, compared with mice treated with 24 mg/kg 5F (Group 2); +$P<0.05$, compared with mice treated with DEN only (Group 4); ++$P<0.01$, compared with mice treated with DEN only (Group 4); ^$P<0.05$, compared with 5F-treated mice at the corresponding doses (Groups 1–3); ^^$P<0.01$, compared with 5F-treated mice at the corresponding doses (Groups 1–3); #$P<0.05$, compared with mice treated with saline only (Group 8) and ##$P<0.01$, compared with mice treated with saline only (Group 8)

prevented the degradation of IkBα induced by TNFα. The level of IkBα was degraded by TNFα in HCC cells in 15–30 min after the treatment, but the degradation was prevented by 5F (Fig. 4b). Second, 5F reduced both basic and TNFα-induced nuclear p65 protein (Fig. 4c), and the effect was confirmed by the decreased activity of NF-kB p65 activity (Fig. 4e). Third, both 5F and Bay 11-7082, a well-known NF-kB inhibitor (21), suppressed Bcl-2 but enhanced Bax and Bak (Fig. 4d), all of which are known-downstream targets of NF-kB (22–24).

## Discussion

The current study is the first one to examine the tumorcidal effect as well as the side-effects of 5F in a HCC mouse model induced by diethylnitrosamine (DEN), a well-known chemical and dietary hepatocarcinogen. DEN-induced HCC in animals is one of the best characterized experimental models of HCC [10, 25]. This model closely resembles the major feature of human HCC [11, 26]. Therefore, it has been widely used to investigate hapatocarcinogenesis and screen

potential anti-HCC compounds [27–31]. Our result clearly indicated that 5F was able to reduce not only the number of DEN-induced liver tumors but also the total volume of tumors in each individual HCC mouse. This finding is of critical importance since the liver of HCC usually contains multiple foci of malignant lesions and the existence of multiple small foci is the most common factor causing the recurrence of HCC. It was also noted that the higher the dose of 5F, the greater the tumorcidal effect, which further supports the effectiveness of 5F as an anti-HCC agent.

During the last several decades, a number of anti-HCC agents have been proposed and tested. However, most of them have failed to reach the clinical stage mainly due to the unbearable side-effects. Even for those that have passed the clinical trials, their use in HCC patients is also often limited in doses and treatment durations, and such limits are justified not only by the tumor itself but by the side-effects generated [32, 33]. Therefore, it is important to develop effective anti-HCC agents with minimal side-effects. During the entire period of the present *in vivo* experiment, the food and water intake, body weights as well as physical activity among 8 groups of mice did not differ significantly. It was
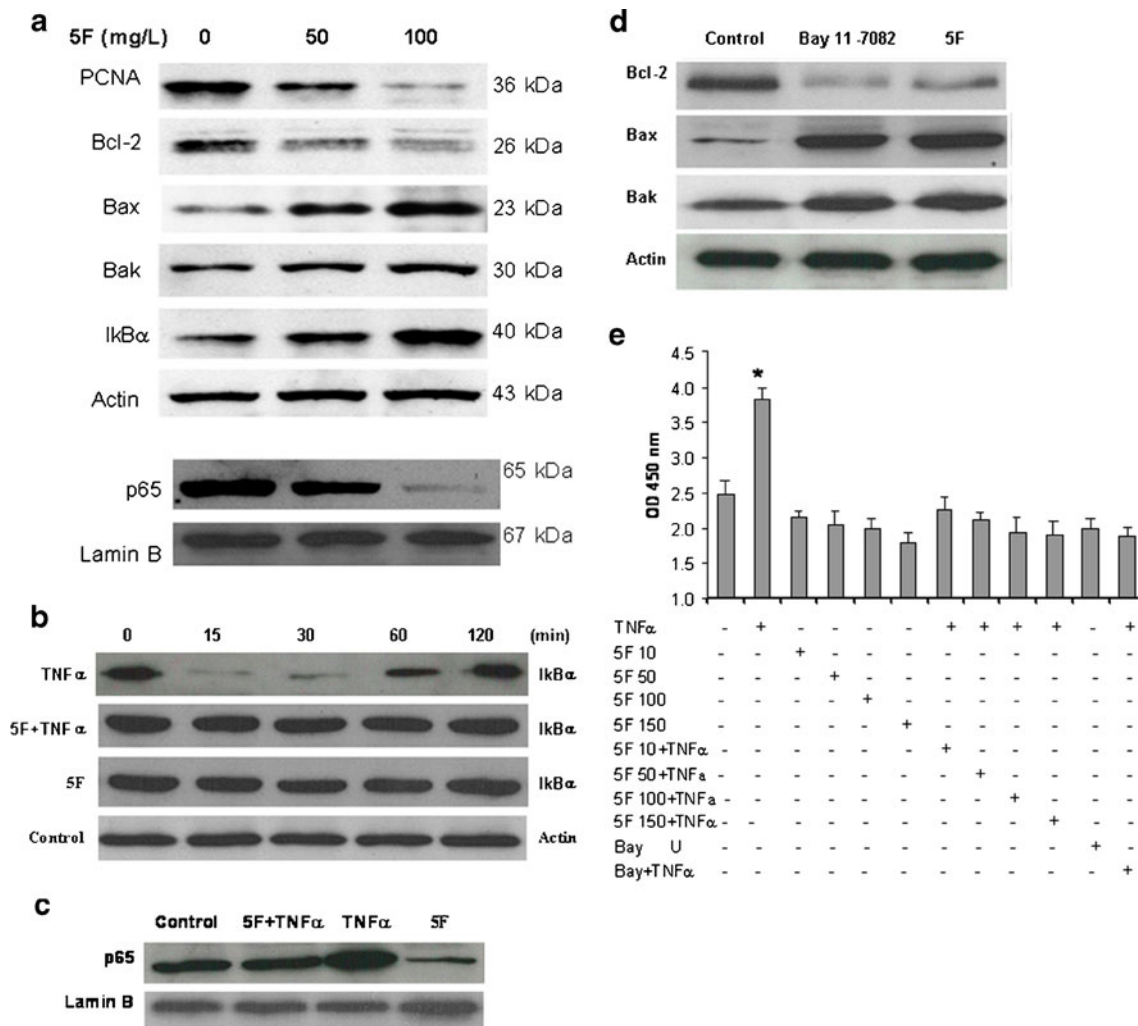
**Fig. 4** Effect of 5F on protein expression in cultured HCC cells. **a** PLC/PFR/5 HCC Cells were treated with 5F at 0, 50 and 100 mg/L for 48 h. At the end of the treatment, the cytoplasmic (PCNA, Bcl-2, Bax, Bak, IkBα) and nuclear (p65) proteins were isolated and subjected to Western blot analysis for the expression of relevant proteins. Equal loading was confirmed by probing with antibodies against actin (cytoplasm) and lamin B (nucleus). **b** PLC/PFR/5 HCC Cells were with 20 ng/ml of TNFα in the presence or absence of 50 mg/L of 5F. Cellular protein was isolated for Western blot of IkBα. Actin was measured as an equal loading control. **c** cells were treated with 5F or vehicle control for 2 h, followed by TNFα or vehicle for 15 min. Nuclear protein was isolated for Western blot of p65. Nuclear protein marker lamin B was measured as an equal loading control. **d** cells were treated with 5F or 10 μM Bay 11-7082 for 48 h. Cellular protein was isolated for Western blot of Bcl-2, Bax and Bak. **e** cells were treated with different doses of 5F or vehicle control for 2 h, followed by TNFα or vehicle for 15 min. Nuclear extracts were prepared for measurement of ELISA of p65 activity as described in Method. $*p<0.01$, compared with all other groups

noted that a mild hair loss was observed in some mice treated with 5F. Fortunately no mice suffered from severe hair loss during or at the end of experiment. These findings indicate that 5F at doses used did not significantly affect the general health of mice. Nevertheless, a further test needs to check whether the hair loss is reversible after ceasing 5F treatment.

The most critical and frequent side-effects generate by anti-tumor agents are the liver and kidney damages. In this study, we demonstrated that while 5F inhibited liver tumor growth, it did not affect liver functions, which were reflected by normal serum levels of AST and ALT, two

well-known markers for the liver function [33, 34]. For the renal function, we examined serum CRE and BUN, which represent the general condition of the kidney [34]. It was found that the levels of CRE in all 8 groups of mice tested were within the normal range. However, the levels of BUN in all 8 groups of mice tested were higher than 7.1 mmol/L, the highest value of the normal range [14]. It is noted that there is not difference in the levels of BUN between the control mice that received saline only and those treated with 5F, indicating that the slight increase of BUN in 5F-treated mice was not caused by 5F treatment. Although our experimental data cannot give an explanation for the slight

increase of BUN, according to the published information there are two possibilities. First, the normal level of BUN in some mouse species can be as high as around 10–13 mmol/L [35, 36]. If we apply this standard to our C3H/HeJ mice, the level of BUN detected in our model should be within the normal range. Second, increased BUN in the setting of a normal serum CRE may not reflect a pathological condition but a physiological response to a relative decrease of blood flow to the kidney. Thus it does not indicate any true damage to the kidney [37]. Furthermore, compared with BUN, serum CRE is a more specific indicator of the renal function [34, 37]. Therefore, the result of BUN does not appear to draw a conclusion that 5F would result in a significant injury to the kidney. Together, our results demonstrate that 5F is not toxic to liver and kidney in the model tested.

Induction of tumor cell death by apoptosis is a major mechanism employed by chemotherapeutic agents and this may be particularly relevant to HCC since the onset of HCC is the result of the imbalance between proliferation and apoptosis in hepatocytes [2]. In the present study, apoptosis in liver tumor cells of 5F-treated mice was determined by the classic TUNEL method and its level was much higher in 5F-treated mice than the mice without 5F treatment. The promotive effect of 5F on apoptosis was further evident by *in vitro* cultured experiments, in which 5F induced apoptosis of HCC cells in a dose-dependent manner. The increase of apoptosis in tumor cells by 5F was in line with the results of decreased PCNA staining and reduced viability of tumor cells. Therefore, the tumorcidal effect of 5F is mainly via the induction of apoptosis.

In order to elucidate the molecular mechanism involved in 5F-mediated induction of apoptosis, we examined the effect of 5F on the expression of NF-kB and Bcl-2 family members. In HCC, NF-kB is constitutively activated and functions as a survival factor in the tumor development [38, 39]. We found that 5F was able to stabilize the NF-kB inhibitor cellular IkBα and prevent it from degradation. The degradation of IkBα is a critical step of NF-kB activation [20]. The failure of IkBα degradation inhibits the NF-kB subunits to translocate to the nucleus, reducing the level/activity of nuclear NF-kB, which is evident in our current study showing the decreased nuclear p65 level and the reduced NF-kB activity. A number of anti- and pro-apoptotic molecules is controlled by NF-kB. Among them are Bcl-2, Bax and Bak. NF-kB can upregulate the anti-apoptotic Bcl-2 by binding to the Bcl-2 promoter whereas it downregulate pro-apoptotic Bax by recruiting repressor complexes to Bak promoter [22, 23]. NF-kB can also inhibit the expression of Bak by promoting Bfl-1, a Bak antagonist [24]. Therefore, these established mechanisms can well explain the decreased level of Bcl-2 but increased levels of Bax and Bak seen in HCC cells treated with 5F. The roles of NF-kB in the regulation of Bcl-2, Bax and Bak in HCC cells

are further verified using a well-known NF-kB inhibitor Bay 11-7082 [21]. Block of NF-kB pathway by Bay 11-7082 leads to reducing Bcl-2 but increasing Bax and Bak in a manner similar to the administration of 5F. The alteration of Bcl-2, Bax and Bak by 5F should be deemed to be significant not only by their roles in the regulation of apoptosis but also the pathological change of these molecules in HCC. The increased level of Bcl-2 and the decreased level of Bax and Bak are known to contribute to the hepatocarcinogenesis or enable HCC cells resistant to anti-tumor treatments [40–45].

In conclusion, 5F is effective against HCC with minimal side effects. It induces apoptosis in HCC cells via stabilizing NF-kB inhibitor IkBα, resulting in the decrease of anti-apoptotic Bcl-2 and increase of pro-apoptotic Bax and Bak.

**Conflict of Interest**   All authors have no conflict of interest.

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