# JIB-04 induces cell apoptosis via activation of the p53/Bcl-2/caspase pathway in MHCC97H and HepG2 cells

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Abstract. JIB-04 is a structurally unique small molecule, known to exhibit anticancer activity and to inhibit the growth of human lung cancer and prostate cancer cell lines. However, the anticancer effect of JIB-04 against human hepatic carcinoma, and its underlying mechanisms, are still unclear. In the present study, MHCC97H and HepG2 cells were employed to investigate the anticancer effects of JIB-04 on cell viability and apoptosis. Annexin V/PI staining, a CCK-8 assay and western blot analysis demonstrated that JIB-04 induced apoptosis in MHCC97H and HepG2 cells, which was evidenced by the expression of proapoptotic and apoptotic proteins including p53, Bak, Bax, caspase-3 and caspase-9. Subsequently, the expression trends of Bcl-2 and p53 were reversed after co-treatment with pifithrin- $\alpha$  (PFT- $\alpha$ , a p53 inhibitor). The results revealed that JIB-04 suppressed the cell viability of MHCC97H and HepG2 cells in a concentration-dependent manner. Meanwhile, it was also demonstrated that JIB-04 effectively triggered MHCC97H and HepG2 cell apoptosis by downregulating Bcl-2/Bax expression, and upregulating proapoptotic and apoptotic protein expression via the p53/Bcl2/caspase signaling pathway. JIB-04 had effects on the inhibition of cell viability and the induction of apoptosis in MHCC97H and

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HepG2 cells. The underlying mechanism of action of JIB-04 was associated with the p53/Bcl-2/caspase signaling pathway. Our findings provide a foundation for understanding the anticancer effect of JIB-04 on MHCC97H and HepG2 cells, and suggested that JIB-04 may be a promising therapeutic agent in human liver cancer.

## Introduction

Liver cancer is a global health issue. Hepatocellular carcinoma (HCC), a major subtype of primary liver cancer, is a one of the most common malignant tumors and is the third leading cause of cancer-related mortality (1,2). HCC develops resistance to most chemotherapeutic agents (3). Significant advances have been made in the early diagnosis and management of HCC; however, therapeutic strategies for the treatment of HCC are still limited (4). At present, surgical techniques remain the most common therapeutic option for the eradication of cancer nodules. Nevertheless, most patients undergoing tumor dissection still suffer from an unsatisfactory outcome, with respect to high recurrence rates and distant organ invasion (5,6). Thus, it can be seen that most HCC patients have a poor prognosis (7). Less than 5% of patients survive for more than 2 years (8). Furthermore, the main anticancer drugs for HCC, such as oxaliplatin and sorafenib, have side-effects and are associated with multidrug resistance (9-11). As an embryonal malignancy of hepatocellular origin, hepatoblastoma (HB) is the most common primary liver tumor in childhood, with a poor prognosis and aggressive behavior (12). Therefore, it is necessary to identify a novel anticancer drug with higher selectivity and greater efficiency against hepatic carcinoma.

JIB-04 is a small molecular compound with two isomers, the E- and Z-isomers (Fig. 1A and B). It is well known that tumors have a complicated and aberrant epigenetic landscape. Only when the cancer epigenome has a certain degree of susceptibility can drugs be targeted for treatment. It was reported that only the E-isomer of JIB-04 was active in a locus de-repression (LDR) assay, which induced the expression of a silenced transgene leading to cancer-specific cell death. The

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results confirmed that JIB-04 could inhibit the growth and metastasis of human lung cancer and prostate cancer cell lines, and thus acted as an antitumor agent (13).

Mutations in the p53 gene have been found in most human malignancies. In addition, mutant p53 proteins can promote tumorigenesis (14-16). As a tumor suppressor existing in most human tumors, p53 protein plays a crucial role in cellular genomic stability and cellular apoptosis after exposure to various types of stress (17,18). It has been demonstrated that p53 is able to regulate the transcription and expression of proapoptotic and apoptotic proteins, including Bak, Bax, caspase-3 and caspase-9, resulting in cellular apoptosis (19,20). Previous studies have demonstrated that cancer cells undergo apoptotic cell death, and that the apoptosis-related caspase-3, caspase-7 and PARP cleavage proteins are activated following stimulation with the E-isomer of JIB-04 (13). However, it remains unknown whether this phenomenon is via a p53 activation-dependent pathway. In addition, the question of whether the E-isomer of JIB-04 displays efficacy in hepatic carcinoma has not been investigated. Therefore, the aim of the current study was to evaluate the anticancer effect of JIB-04, and to explore the underlying mechanism of JIB-04 induced-apoptosis in MHCC97H and HepG2 cells.

#### Materials and methods

Medicine and reagents. JIB-04 E-isomer (C<sub>17</sub>H<sub>13</sub>ClN<sub>4</sub>; Fig. 1) was purchased from Sigma-Aldrich (cat. no. SML0808; Merck KGaA, Darmstadt, Germany), and then was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution at a concentration of 50 mM. All stock solutions were stored at -20°C in the freezer. The working solutions of JIB-04 were prepared by further diluting the stock solutions with culture medium. The final concentration of DMSO was below 0.1% in this study. Pifithrin- $\alpha$  (PFT- $\alpha$ ) was obtained from Sigma-Aldrich (cat. no. P4359; Merck KGaA) and diluted to a final concentration of 30  $\mu$ M. The Cell Counting Kit-8 (CCK-8) (cat. no. CK04) and the Annexin V-FITC apoptosis detection kit (cat. no. AD10) were obtained from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). The primary antibodies against caspase-3 (cat. no. 9665S), caspase-9 (cat. no. 9502S), Bak (cat. no. 3814S), Bcl-2 (cat. no. 4223S), Bax (cat. no. 2772S), p53 (cat. no. 9282S), PARP (cat. no. 5542L) and GAPDH (cat. no. 2118L) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The secondary antibody was obtained from Sino Biological, Inc. (cat. no. SSA004; Beijing, China).

Cell lines and drug treatment. The MHCC97H and HepG2 cells, obtained from the cancer cell repository (Shanghai Cell Bank, Shanghai, China), were cultured in DMEM (cat. no. 21885108; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (cat. no. 10099141; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin at 37°C in a humidified incubator with an atmosphere of 5% CO<sub>2</sub>. Both MHCC97H and HepG2 cells were treated with various concentrations (0, 0.25, 0.5 and 1  $\mu$ M) of JIB-04 when the cell confluence reached 70-80%.



Figure 1. Chemical structures of JIB-04 E (A) and Z (B) isomers.

Cell inhibition and cytotoxicity assay. CCK-8 assay: Cells were seeded at a density of  $5x10^3$  cells/well into 96-well plates. Briefly, different concentrations of JIB-04 were used to treat MHCC97H and HepG2 cells. Following various exposure times, the supernatants were removed. CCK-8 solution was diluted 10 times in warm assay medium. Then, 100  $\mu$ l diluent was transferred to each well. Plates were incubated for 2 h at 37°C with 5% CO<sub>2</sub>. The absorbance was recorded by using a plate reader (Perkin-Elmer, Inc., Waltham, MA, USA) at a wavelength of 450 nm. The experiments were performed independently and at least in triplicate. Inhibition rate (%) was calculated according to the following equation: Inhibition rate (%) = [OD<sub>450(control)</sub> - OD<sub>450(treated)</sub>]/[OD<sub>450(control)</sub> - OD<sub>450(blank)</sub> x 100%.

Assessment of cell morphology. Cells were seeded at a density of  $1 \times 10^5$  cells/well into a 6-well plate. After pretreatment with different concentrations (0, 0.25, 0.5 and 1  $\mu$ M) of JIB-04 for different exposure times (48 h for MHCC97H and 72 h for HepG2), images were captured (scale bar, 200  $\mu$ m) by an inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Apoptosis assay. Apoptotic cells were detected using an Annexin V-FITC apoptosis detection kit (Dojindo Molecular Technologies, Inc.). Experiments were carried out using flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA) and analyzed using ModFit and CellQuest 5.1 software (BD Biosciences). According to the manufacturer's instructions, MHCC97H and HepG2 cells were plated at an initial concentration of 1x10<sup>5</sup> cells/well in 6-well plates. After incubation for 12 h, the cells were treated with different concentrations (0, 0.25, 0.5 and 1  $\mu$ M) of JIB-04 for various exposure times (48 h for MHCC97H and 72 h for HepG2), and were then harvested and washed twice with cold D-Hanks buffer solution. The cells were resuspended in binding buffer (1x10<sup>6</sup> cells/ml). Subsequently, 5  $\mu$ l Annexin V-FITC and 5  $\mu$ l propidium iodide (PI) were added to 100  $\mu$ l cell supernatant, and incubated in the dark for 10 min prior to analysis. The Annexin V-positive cells were regarded as being in the early apoptosis stage, while Annexin V and PI double-positive cells were in the late apoptosis stage.



Figure 2. JIB-04 inhibits MHCC97H and HepG2 cell viability. JIB-04 induced apoptosis in (A) MHCC97H cells following treatment at concentrations of 0, 0.25, 0.5 and 1  $\mu$ M for 48 h, and (B) in HepG2 cells for 72 h, which was visualized by microscopy (scale bar, 200  $\mu$ m). Quantification of CCK-8 assay data for (C) MHCC97H and (D) HepG2 cells. Values are presented as the mean ± standard deviation (n=3). \*P<0.05 and \*P<0.01 compared with the 24 h group.

Western blot analysis. The MHCC97H and HepG2 cells were cultured at an initial concentration of 1x10<sup>5</sup> cells/ml in 100 mm culture dishes, and incubated for 12 h at 37°C for 24 h. Subsequently, cells were pretreated with different concentrations (0, 0.25, 0.5 and 1  $\mu$ M) of JIB-04 for different exposure times (48 h for MHCC97H and 72 h for HepG2) and lysed in lysis buffer (cat. no. P0013; Beyotime Institute of Biotechnology, Haimen, China) for 30 min on ice. The lysates were centrifuged at 10,391.81 x g at 4°C for 10 min. Then, the supernatants were collected. The protein expression levels of samples were measured using a BCA concentration measurement kit (cat. no. P0010; Beyotime Institute of Biotechnology). Lysates were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and were then transferred to polyvinylidene difluoride membranes (PVDF; EMD Millipore, Billerica, MA, USA). Blocking was performed with 5% fat-free dry milk in Tris-buffered saline (cat. no. V900483; Sigma-Aldrich, Co., Merck KGaA) containing 0.05% Tween-20 (TBST) for 1 h. Subsequently, the membranes were incubated with the primary antibodies, including anti-caspase-3, anti-caspase-9, anti-Bak, anti-Bcl-2, anti-p53, anti-Bax, anti-PARP and GAPDH, at a dilution of 1:1,000 overnight at 4°C. Following washing three times with TBST, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG at a dilution of 1:2,000 for 4 h at 4°C. The membranes were washed with TBST three times for 10 min each. Detection was carried out using the enhanced chemiluminescence method. The integrated density value of each band for each image was calculated using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. All data are expressed as the mean  $\pm$  standard deviation (SD). Statistical analysis was performed using SPSS software (version 17.0; IBM Corp., Armonk, NY, USA). One-way ANOVA (Tukey's test) was used to evaluate between-group differences. Statistical significance was defined as P<0.05.

#### Results

JIB-04 inhibits cell proliferation in MHCC97H and HepG2 cells. In order to investigate the antitumor effects of JIB-04, MHCC97H and HepG2 cells were treated with 0, 0.25, 0.5 and 1  $\mu$ M of JIB-04 for 24, 48 and 72 h and cell viability was determined using a CCK-8 assay. It was found that untreated cells grew well; however, treated cells were distorted and rounded in shape. In addition, the number of floating cells increased significantly as the drug concentration increased. As can be seen from Fig. 2, the results demonstrated that JIB-04 inhibited the cell viability of MHCC97H and HepG2 cells in a concentration-dependent manner.



Figure 3. JIB-04 induces MHCC97H and HepG2 cell apoptosis. The apoptosis rate in (A) MHCC97H and (B) HepG2 cells following treatment with 0, 0.25, 0.5 and 1  $\mu$ M of JIB-04 for 48 and 72 h. Apoptotic cells were detected using flow cytometry. Quantification of the apoptosis rate data for (C) MHCC97H and (D) HepG2 cells determining the rates of early and late apoptosis. Values are presented as the mean ± standard deviation (n=3). \*P<0.05 and \*\*P<0.01 compared with 0  $\mu$ M JIB-04.

JIB-04 induces apoptosis in MHCC97H and HepG2 cells. Annexin V/PI double staining was used to detect cellular apoptosis. Compared with the vehicle-treated control, the Q2 and Q4 cell population in the MHCC97H and HepG2 cells, respectively increased from 5.57 to 46.05% (48 h), and from 6.85 to 48.3% (72 h). Fig. 3 shows that JIB-04 induced cellular apoptosis in a concentration-dependent manner.

JIB-04 induces the expression of p53/Bcl-2/caspasesignaling pathway proteins in MHCC97H and HepG2 cells. The p53/Bcl-2/caspase signaling pathway is tightly associated with cell apoptosis. In this study, MHCC97H and HepG2 cells were treated with 0, 0.25, 0.5 and 1  $\mu$ M JIB-04 for different exposure times and the expression levels of p53/Bcl-2/caspase signaling pathway proteins were evaluated. Fig. 4 shows that apoptosis-related protein expression altered following JIB-04 stimulation. After MHCC97H and HepG2 cells were treated with different concentrations of JIB-04, the expression levels of the apoptosis-related proteins p53, caspase-3 and caspase-9 were significantly upregulated in a dose-dependent manner. As an important substrate of caspase-3, PARP was found to increase in the JIB-04-treated cells compared with the controls. In addition, Bcl-2 expression was downregulated. Bax was significantly upregulated in HepG2 and MHCC97H cells after treatment with increasing concentrations of JIB-04. Therefore, the Bcl-2/Bax protein ratio in MHCC97H and HepG2 cells was decreased in a concentration-dependent manner. The results indicated that intrinsic apoptosis pathways, including the caspase-9/caspase-3-associated apoptosis pathway and the p53/Bcl-2 signaling pathway, were activated following JIB-04 treatment in MHCC97H and HepG2 cells.

p53 plays an important role in JIB-04-induced apoptosis in MHCC97H and HepG2 cells. In order to determine the role of p53 in JIB-04-induced cellular apoptosis, MHCC97H and HepG2 cells were pretreated with PFT- $\alpha$  (30  $\mu$ M) for 6 h in the presence or absence of JIB-04 (0.5  $\mu$ M). As can be seen from Figs. 5-7, JIB-04 triggered cellular apoptosis in MHCC97H and HepG2 cells; however, PFT- $\alpha$  reversed JIB-04-induced cell growth suppression and apoptosis, and significantly reduced the expression level of p53, Bax, cleaved caspase-3 and -9 protein. Simultaneously, Bcl-2 expression was upregulated. The results demonstrated the key role of p53 in JIB-04-induced cellular apoptosis in MHCC97H and HepG2 cells.



Figure 4. JIB-04 regulates the p53/Bcl-2/caspase signaling pathway. JIB-04 regulated the expression of apoptosis-related proteins in MHCC97H and HepG2 cells following treatment with 0, 0.25, 0.5 and 1  $\mu$ M of JIB-04 for 48 and 72 h. (A) Western blotting was carried out to evaluate the expression levels of p53, Bcl-2, Bax, Bak, PARP and caspase-3 and -9. (B) Integrated density data were quantified. All images are representative of three independent experiments. Values are presented as the mean ± standard deviation (n=3). \*P<0.05 and \*\*P<0.01 compared with 0  $\mu$ M JIB-04.



Figure 5. Pifithrin- $\alpha$  (PFT- $\alpha$ ) reverses JIB-04-induced MHCC97H and HepG2 cell apoptosis. The apoptosis rate in (A) MHCC97H and (B) HepG2 cells following co-treatment with 0.5  $\mu$ M JIB-04 or 30  $\mu$ M PFT- $\alpha$  for 48 and 72 h. Apoptotic cells were detected using flow cytometry. Quantification of the apoptosis rate data for (C) MHCC97H and (D) HepG2 cells determining the rates of early and late apoptosis. Values are presented as the mean  $\pm$  standard deviation (n=3). \*P<0.05 and \*\*P<0.01 compared with JIB-04 (+) PFT- $\alpha$  (-).

## Discussion

Despite advances in therapeutic strategies for HCC, such as transplantation, immunotherapy strategies and liver resection, the 5-year overall survival rate remains poor (21-23). Thus, the identification of novel anticancer drugs with higher therapeutic efficacy against HCC is a key focus in oncology research. JIB-04, a potential therapeutic agent against cancer, has been confirmed to inhibit the viability of multiple cancer cell lines (13). However, its underlying mechanism and its efficacy in inhibiting hepatoma cell growth *in vitro* is not clear. In this study, JIB-04 was demonstrated to reduce the proliferation of MHCC97H and HepG2 cells in a dose-dependent manner, indicating that JIB-04 may serve as a novel candidate agent against hepatic carcinoma.

Apoptosis, a complicated physiological process, involves complex signaling pathways, such as the activation of cysteine proteases and p53 (24,25). Apoptosis induction has been accepted as a mechanism of action of anticancer drugs (26). At present, the development of anticancer agents is mainly focused on inducing apoptosis in tumor cells (27). Using Annexin V/PI staining, the present study demonstrated that JIB-04 induced MHCC97H and HepG2 cell apoptosis in a concentration-dependent manner, indicating that apoptosis may serve as a mechanism underlying the function of JIB-04.

In order to further explore the underlying mechanism of JIB-04-induced apoptosis in MHCC97H and HepG2 cells, we evaluated the common regulators of the apoptosis process. Cysteine proteases, especially caspases, play a crucial role in regulating cellular apoptosis (24). Owing to their different mechanisms of action, caspases are divided into initiator caspases, including caspase-8 and -9, and executioner caspases, including caspase-3, -6 and -7 (28). Caspase-3 is mainly activated through two signaling pathways: the extrinsic pathway, involving the activation of death receptors and caspase-8; and the intrinsic pathway, involving the mitochondria and activation of caspase-9, which then results in cellular apoptosis (29,30). In this study, the results demonstrated that cleaved caspase-3 and caspase-9 were significantly upregulated after JIB-04 stimulation, indicating that JIB-04 induced apoptosis in the MHCC97H and HepG2 cells through the mitochondrial-mediated apoptosis pathway. As a molecular receptor of DNA damage, PARP is activated and participates in DNA replication and transcription when DNA is damaged (31,32). In this study, the results showed that PARP was cleaved significantly, suggesting that JIB-04 inhibited MHCC97H and HepG2 cell growth via cleaved caspase-3 and PARP.



Figure 6. JIB-04 induces MHCC97H and HepG2 cell apoptosis via the p53 signaling pathway. (A) JIB-04-induced HCC cell apoptosis was reversed following co-treatment with 0.5  $\mu$ M JIB-04 or 30  $\mu$ M pifithrin- $\alpha$  (PFT- $\alpha$ ) for 48 and 72 h. Images were captured by microscopy (scale bar, 200  $\mu$ m). (B) Western blotting was performed to investigate the correlation between p53 and apoptosis-related proteins (Bcl-2 and Bax) in HCC cells after PFT- $\alpha$  treatment.



Figure 7. JIB-04-induced HCC cell apoptosis was reversed following co-treatment with  $0.5 \,\mu$ M JIB-04 or  $30 \,\mu$ M pifithrin- $\alpha$  (PFT- $\alpha$ ) for 48 and 72 h. Western blotting was carried out to evaluate the expression levels of p53, Bcl-2, Bax and caspase-3 and -9. Integrated density data were quantified. (A) MHCC97H. (B) HepG2. All images are representative of three independent experiments. Values are presented as the mean ± standard deviation (n=3). \*P<0.05 and \*\*P<0.01 compared with JIB-04 (+) PFT- $\alpha$  (-).

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Apoptosis is not only associated with the activation of caspases, but the accumulation of apoptosis-related proteins (Bcl-2 family) (33). To the best of our knowledge, the apoptosis-suppressing function of Bcl-2 is inhibited after binding with Bax protein. The apoptosis-inducing effects are more associated with the ratio of Bcl-2/Bax than with the quantity of Bcl-2 alone (34,35). A previous study demonstrated that the p53/Bcl-2 pathway was closely related to dihydromyricetin-induced HCC cell apoptosis (25). In the present study, JIB-04 induced MHCC97H and HepG2 cell apoptosis, which was verified by the downregulation of the ratio of Bcl-2/Bax and upregulation of p53 protein expression via the p53/Bcl-2 signaling pathway (36). Bak, a core regulator of the intrinsic apoptosis pathway, was significantly upregulated following JIB-04 stimulation. These results confirmed that JIB04 inhibited cell growth and induced the apoptosis of MHCC97H and HepG2 cells.

Although many proteins are directly involved in the regulation of p53 levels and functioning, it is generally accepted that MDM2 is the principal negative regulator of p53 (37). Serving as an E3 ubiquitin ligase of p53, MDM2 not only negatively regulates p53 activity through the induction of p53 protein degradation (38), but directly inhibits p53 trans-action on chromatin (39). In summary, MDM2 can repress p53 via both ubiquitination-dependent and ubiquitination-independent pathways. Furthermore, MDMX can negatively regulate the stability and activity of the p53 protein by mediating the rapid degradation of p53 via ubiquitin-dependent proteolysis (38,40). As a tumor-suppressor gene, p53 can induce apoptosis by mediating several classical pathways (41). It is considered a suitable choice for the treatment of various tumors by targeting and reactivating p53 or enhancing its activity (42). In this study, when JIB-04 was blocked by PFT- $\alpha$ , Bcl-2 suppression was reversed significantly by decreasing p53 expression. Subsequently, the results verified that JIB-04 induced MHCC97H and HepG2 cell apoptosis via the p53/Bcl-2 pathway.

In conclusion, the present study demonstrated that JIB-04 effectively inhibited cell viability, and promoted MHCC97H and HepG2 cell apoptosis via the p53/Bcl2/caspase signaling pathway. The results provided a foundation for understanding the anticancer effect of JIB-04 on MHCC97H and HepG2 cells, and suggest that JIB-04 may be a promising compound for the treatment of hepatic carcinoma.

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# Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

#### Authors' contributions

Conception and design was carried out by ML and RZ. Administrative support was undertaken by RZ. Provision of study materials was performed by WL and JL. All authors aided in the performance of the experiments. Collection and assembly of data was achieved by BL and DC. Data analysis and interpretation was carried out by XH, YY and WL. Manuscript writing was undertaken by WL. All authors were involved in the final approval of the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

#### Ethics approval and consent to participate

Not applicable.

#### Patient consent for publication

Not applicable.

#### **Competing interests**

The authors declare that they have no competing interests.

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