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Research article

Compound Taxus exerts marked anti-tumor activity and radiosensitization effect on hepatocellular carcinoma cells

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ABSTRACT

Background: Compound Taxus capsule, as an antineoplastic Chinese patent drug, has been increasingly applied as an adjunctive treatment for the management of non-small-cell lung cancer (NSCLC) and some other malignancies, but research about its antitumor activity and radiosensitization effect on hepatocellular carcinoma (HCC) cells is very rare.

Purpose: To investigate the antitumor activity and radiosensitization effect of Compound Taxus on HCC cells and to preliminarily explore the possible molecule mechanisms involved.

Methods: Cell viability, cell cycle distribution, apoptosis, DNA damage repair and protein expression levels were detected by CCK-8 assay, flow cytometry, immunofluorescence staining, western blotting analysis and immunohistochemical staining, respectively. The migration and invasion activities and vasculogenic mimicry (VM) formation and angiogenesis were evaluated by tube formation and VM formation assay. Radiation survival curves were obtained from the colony formation assay in human HCC cell lines, Smmc7721 and Bel7402 cells, pretreated with or without Compound Taxus before receiving X-ray irradiation. A Bel7402 tumor-bearing mouse model was established and the radiosensitization effect of Compound Taxus in vivo was evaluated by analyzing tumor volume and tumor weight in different groups receiving different treatments.

Results: Compound Taxus decreased viability, induced G2/M arrest, promoted apoptosis, suppressed migration and invasion, and inhibited VM formation and angiogenesis in Smmc7721 and Bel7402 cells. Furthermore, Compound Taxus inhibited irradiation-induced DNA damage repair, enhanced the radiosensitivity of Smmc7721 and Bel7402 cells and improved the anti-tumor therapeutic efficacy of irradiation in Bel7402 tumor-bearing mice. Radiotherapy in combination with Compound Taxus showed the best tumor inhibition compared to that of Compound Taxus alone or irradiation alone. In addition, Compound Taxus significantly down-regulated NF-κB p65, p–NF–κB p65 and Bcl-2, and up-regulated Bax in vitro and in vivo, yet NF-κB p65 overexpression reversed the proapoptotic effect of Taxus on HCC cells, indicating that the NF-κB signaling pathway might be an important signal mediator in the Compound-Taxus-modulated biological responses.

Conclusion: Our findings suggest that Compound Taxus shows marked antitumor activity and significant radiosensitization effect on HCC cells, making it possible for Compound Taxus to

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become a promising auxiliary modality for HCC management and a potential radiosensitizer of HCC in the future.

1. Introduction

Hepatocellular carcinoma (HCC) is a kind of extraordinarily heterogeneous malignancy, and its incidence and mortality are increasing gradually [1]. It is now the fifth most common human cancer [2] and the second cause of cancer-related mortality worldwide [3]. The treatment modalities for HCC vary according to factors such as tumor stage and location, including surgery, chemotherapy, radiotherapy (RT), and radiofrequency ablation. However, most HCC patients were dianosed too late to be eligible for surgical resection, owing to lack of obvious symptoms in the early stages [4]. RT is essential for patients who are unfit for resection [5]. As one of the important local treatment methods for HCC, RT has been increasingly applied in clinical practice [6,7], but the curative effect still needs to be improved. In light of this, extraordinary efforts are needed to be devoted to the study of improving the therapeutic ratio of cancers to RT by using radiosensitizers [8]. Low-toxicity and high-efficiency drugs are ideal as radiosensitizers [9]. During recent years, researchers have been focusing on naturally occurring compounds that show antitumor effects with limited collateral issues [10].

Compound Taxus capsule is a kind of oral antitumor Chinese patent medicine which is comprised of Taxus chinensis, red ginseng and glycyrrhiza uralensis. In China, Compound Taxus capsule was approved as an adjuvant therapy to treat lung cancer of middle and advanced stage, and in some reports it was also used to treat ovarian, liver, cervical, esophagus, gastric and nasopharyngeal cancers. Taxus chinensis has a long history of use as ethnomedicine in oriental and western countries [11]. Studies have found that Taxus extracts have many biological functions, including anticonvulsant, antipyretic, antihyperglycemic, anti-inflammatory, antioxidant and analgesic activities [12–14]. The anticancer activity of Taxus extracts in different human cancer cell lines and the synergistic effect with 5-fluorouracil were certified in previous studies [15,16]. In addition, red ginseng in compound Taxus capsule contains ginsenosides, various amino acids and other components, which was found to have the function of improving immunity [17–19]. Glycyrrhiza uralensis might be used for cancer therapy to ameliorate the side effects of chemotherapy and enhance the immunity [20–22]. In this study, we first prepared Compound-Taxus-containing serum using SD rats and investigated the effects of Compound Taxus on cell viability, cell cycle, apoptosis, migration and invasion, VM formation and angiogenesis, DNA damage repair and radiosensitivity of HCC cells in vitro and in vivo, and preliminarily explored the possible mechanisms involved. To our knowledge, this is the first report about the radiosensitization effect of Compound Taxus on HCC cells.

2. Materials and methods

2.1. Cell culture

HCC cell lines (Smmc7721 and Bel7402) were kindly provided by Dr. Jingyi He (Shandong University). The above cells were cultured in RPMI-1640 (Gibco, USA) medium containing 10% FBS (Hyclone) and 1% penicillin-streptomycin (Hyclone). Cells were maintained in humid CO2 incubator at 37 °C.

2.2. Drug preparation

For in vitro experiments, Compound-Taxus-containing serum was prepared. Briefly, SD rats were randomly divided into two groups, control group and Compound-Taxus-containing serum group. The dose was the recommended human dose × 6.2 mg/kg. Saline and Compound Taxus capsule (Chongqing Sainuo, China) (0.159 g/kg, once a day for 14 days) were administered intragastrically, respectively. Two hours after the last intragastric administration, blood was collected through aorta abdominalis and centrifuged. Serum was filtered and sterilized, and stored at −20 °C for future use [23]. For in vivo treatment, Compound Taxus was dissolved in saline.

2.3. Cell viability assay

Appropriate amounts of Smmc7721 and Bel7402 cells were seeded into 96-well plates and treated with different doses of Compound-Taxus-containing serum for 48 h. The cytotoxicity of Compound Taxus was evaluated by Cell Counting Kit-8 assay (CCK-8) (Dojindo). Briefly, 10 μl of CCK8 was added to each well and after 2 h' incubation the absorbances at 450 nm were recorded. For each group, five replicate wells were included, and three independent experiments were performed. Cell viability was calculated by the equation provided by the manufacturer.

2.4. Wound healing assay

Smmc7721 and Bel7402 cells were seeded into 6-well plates and incubated to reach more than 90% confluence. The cell layers were scratched using a disinfected pipette tip to create wounds. Then, cells were cultured in serum-free medium with a specific concentration of Compound-Taxus-containing serum. Images of wound healing were obtained by an inverted microscope, and the healing rate

was calculated using Image J software (National Institutes of Health).

2.5. Transwell assay

Appropriate amounts of Smmc7721 and Bel7402 cells suspended in serum-free medium (150 μl) with different concentrations of Compound-Taxus-containing serum were added in the top chamber (24-well, membrane with 8 μm pores, Corning Incorporated, USA). Then add 600 μl of medium with 20% FBS into the lower chamber. For cell invasion assay, chamber membranes were pre-coated with Matrigel (BD Biosciences, USA). After incubation for 24 h, cells on the upper side of the membrane were wiped with a cotton swab, and cells on the bottom side of the membrane were fixed in 4% paraformaldehyde, stained with crystal violet (KeyGEN, China) and counted. Images were captured in five different fields.

2.6. Tube formation assay

Matrigel (BD, USA) was dissolved at 4 °C for 24 h and then injected into 96-well plates (70 μL/well). 1×10^4 HUVECs in 50 μL of tumor-cell-conditioned medium were seeded and incubated at 37 °C for 2 h. Images were captured every 2 h and the total numbers of branches, junctions, and segments were calculated using ImageJ.

2.7. VM in vitro

Smmc7721 and Bel7402 cells (8×10^3 cells/well) were seeded into Matrigel-coated 96-well plates with Compound-Taxus-containing serum (0, 5%, and 10%) and cultured at 37 °C for 24 h. VM tubule structures in five different fields were viewed and captured by phase-contrast microscope. The average number of master junctions and total segment length were analyzed using ImageJ.

2.8. Radiation and colony formation assay

Smmc7721 and Bel7402 cells were pretreated with 0 or 5% Compound-Taxus-containing serum for 12 h and then exposed to 0–10Gy irradiation (652.69 cGy/min) with precision x-ray irradiator (X-RAD 225, USA) kept on ice. 12 h after irradiation, culture supernatants were replaced with fresh medium and cells were incubated for additional 14 days. Subsequently, colonies were washed, fixed, stained, and then counted under a dissecting microscope. Only those colonies with over 50 cells were included to establish the radiation survival curve [24].

2.9. Cell transfection

Smmc7721 and Bel7402 cells were seeded into 6-well plates and transfected with pcDNA3.1-p65 or the empty vector with Lipofectamine 3000 Reagent (Invitrogen, USA). 48 h post-transfection, the cells were pretreated with 0 or 5% Compound-Taxus-containing serum for 12 h before X-ray irradiation (4Gy), and then collected for subsequent experiments.

2.10. Apoptosis assay

Smmc7721 and Bel7402 cells were seeded into 6-well plates and treated with Compound-Taxus-containing serum for 24 h. As was previously described [25], cells were then collected, washed twice with precooled phosphate-buffered saline (PBS) and resuspended in 500 mL binding buffer. After adding 5 μl of Annexin V-FITC and 5 μl of propyl propionate (BD, USA), the cells were kept out of light and incubated at room temperature (RT) for 15 min and then underwent flow cytometry (BD, USA). Results were analyzed with FlowJo 7.6.2 software (BD, USA).

2.11. Cell cycle analysis

Appropriate amounts of Smmc7721 and Bel7402 cells were seeded into 6-well plates and treated with Compound-Taxus-containing serum for 12 h. Cells were subsequently collected, washed with PBS, resuspended in prechilled 75% ethanol and kept overnight at 4 °C. The next day cells were re-washed with PBS, and resuspended in double distilled water with addition of 1 mg/mL RNase A. After an incubation for 40 min at 37 °C, the cells were stained with PI solution in the darkness for 20 min and then analyzed by flow cytometry.

2.12. Western blotting analysis

Smmc7721 and Bel7402 cells were seeded into 6-well plates and treated with Compound-Taxus-containing serum for 24 h. As was described by other studies [24], cells were collected and lysed with radioimmunoprecipitation assay lysis buffer, and protein concentrations were detected using the BCA method. Equal amounts of protein (30 μg/lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene difluoride membranes (Solarbio). After blocking with 5% nonfat milk at RT for 1 h, the filters were incubated with specific primary antibodies at 4 °C overnight. The next day after washing and incubating with horseradish peroxidase (HRP)-conjugated secondary antibodies at RT for 1 h, the immune

complexes were visualized by the electrochemiluminescence system. Immunoblots were quantified by ImageJ and the intensity levels were normalized to the internal control Glyceraldehyde-3-phosphate dehydrogenase (Gapdh).

2.13. Immunofluorescence staining

Smmc7721 and Bel7402 cells were cultured on coverslips in 24-well plates for 24 h and treated with Compound-Taxus-containing serum. Some cells were treated with Compound-Taxus-containing serum for 24 h, and then NF-κB p65 immunofluorescence assay was performed. Other cells were pretreated with 0 or 5% Compound-Taxus-containing serum for 8 h and then irradiated by 4 Gy X-rays. Rad51 foci immunofluorescence assay was performed 12 h after irradiation. Briefly, Cells were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100, blocked with 5% bovine serum albumin for 15 min at RT, and incubated with specific primary antibody overnight at 4 °C. The next day after washing with PBS for 3 times, cells were incubated with the Alexa Fluor 488-conjugated secondary antibody for 1 h and nuclei were stained with DAPI [3]. Images were captured by a fluorescent microscope (Olympus, Japan). And NF-κB p65 levels were semiquantitatively evaluated by ImageJ.

2.14. Establishment of a Bel7402 tumor-bearing mouse model and evaluation of therapeutic efficacy of different treatments

All the animal experiments were approved by the Medical Ethics Committee of Shandong Provincial Hospital and performed in accordance with the relevant guidelines and regulations. 1×10^7 Bel7402 cells mixed with standard Matrigel were inoculated into the right thighs of 5-week-old male nude mice. When the mean tumor volume reached 50 mm^3, all mice were divided into the following four groups at random (n = 5): control (Con), Compound Taxus (CT), X-ray irradiation (RT) and combination (Comb) group. 300 mg/

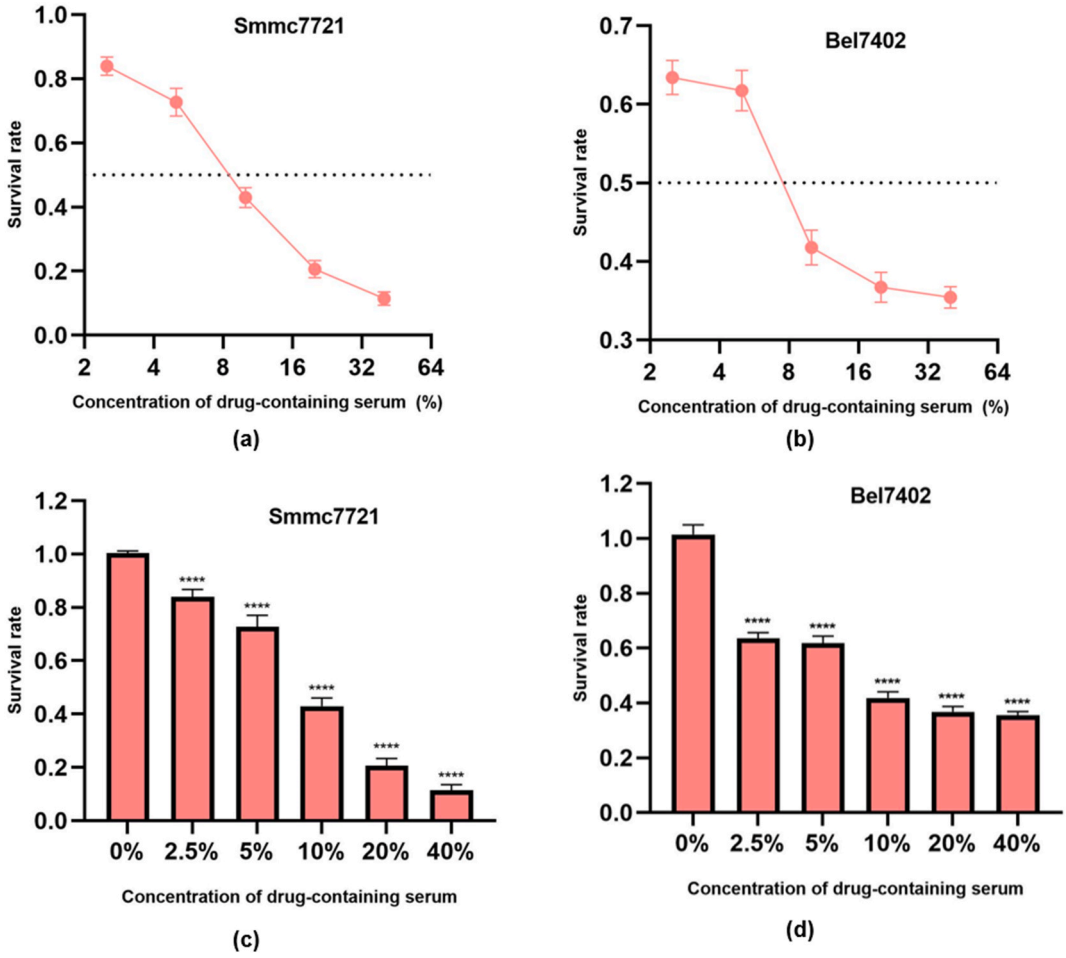

Fig. 1. Compound Taxus decreased viability of Smmc7721 and Bel7402 cells. (a,b) Smmc7721 and Bel7402 cells of logarithm growth period were incubated with different concentrations of Compound-Taxus-containing serum for 24 h, then cell viability was determined by CCK-8 assay. The IC50 of Compound-Taxus-containing serum in Smmc7721 and Bel7402 cells were (8.938 ± 0.444)% and (7.948 ± 0.190)%, respectively. (c,d) Compared with the control group, Compound Taxus treatment significantly decreased the survival rate of both Smmc7721 and Bel7402 cells. Values are represented (all data are expressed) as the mean ± SD. The experiment was repeated at least three times. ****$p < 0.0001$.

kg/day Compound Taxus and saline were given intragastrically to CT and Con groups for 14 days, respectively. The RT and Comb groups received a single dose of 8 Gy X-ray irradiation. The Comb group were given the first dose of Compound Taxus 2 h before X-ray irradiation and received other doses of Compound Taxus from the next day. Tumor volume and weight were periodically monitored to evaluate the therapeutic efficacy. Tumor volume was calculated according to the formula: length $\times$ width^2 $\times$ 0.523 [24,26].

2.15. Immunohistochemical staining

The tumor samples were fixed with 4% paraformaldehyde and embedded in paraffin for slicing. Immunohistochemical staining of NF-κB p65, Bax, and Bcl-2 was carried out in the tumor slices. Briefly, the slices were deparaffinized, rehydrated and antigen retrieved. Subsequently, they were incubated with specific primary antibodies (anti–NF–κB p65, anti-Bax and anti-Bcl-2 (Abcam)) at RT for 30 min. Then they were washed and incubated with a HRP-labeled secondary antibody. After washing, the slices were stained in diaminobenzidine solution, counterstained in hematoxylin, and observed under a microscope.

2.16. Statistical analysis

Data analysis was performed using Prism 8. Data are presented as the mean ± SD of at least three independent experiments. Continuous data between two groups were compared using Students' t-test, and differences among groups were determined with 1-way or 2-way analysis of variance, followed by Tukey's post hoc test. A probability value of 0.05 was considered statistically significant.

3. Results

3.1. Compound Taxus decreased viability of Smmc7721 and Bel7402 cells

The effect of Compound Taxus on viability of Smmc7721 and Bel7402 cells were determined by CCK-8 assay after 24 h of Compound-Taxus-containing serum treatment. The IC50 of Compound-Taxus-containing serum in Smmc7721 and Bel7402 cells were (8.938 ± 0.444)% and (7.948 ± 0.190)%, respectively (Fig. 1(a) and (b)). Compared with the control group, Compound Taxus treatment significantly decreased the survival rate of both Smmc7721 and Bel7402 cells (Fig. 1(c) and (d)).

3.2. Compound Taxus induced G2/M phase arrest in Smmc7721 and Bel7402cells

To explore the effect of Compound Taxus on cell cycle, Smmc7721 and Bel7402 cells were treated with different concentrations of

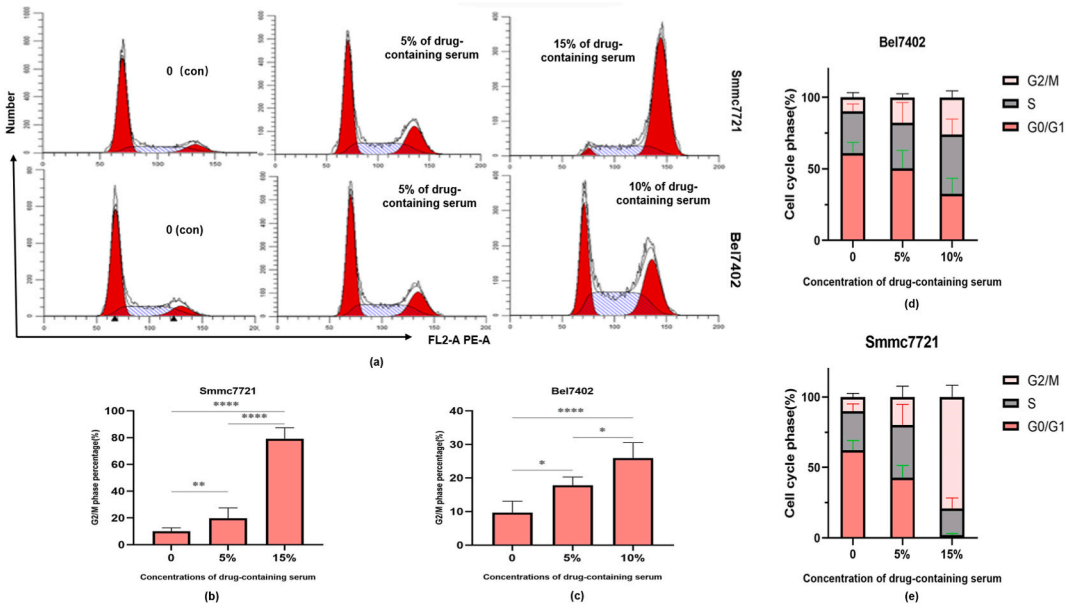

Fig. 2. Compound Taxus induced G2/M phase arrest in Smmc7721 and Bel7402 cells. Smmc7721 and Bel7402 cells were incubated with Compound-Taxus-containing serum (0, 5% or 15% for Smmc7721 cells and 0, 5% or 10% for Bel7402 cells) for 12 h and then analyzed by flow cytometry. (a) Representative results of cell cycle changes from three independent experiments. (b,c) Quantitative analysis of G2/M phase percentage. (d,e) The presentation of the entire cell cycle phases. Values are represented (all data are expressed) as the mean ± SD. The experiment was repeated at least three times. *$p < 0.05$, **$p < 0.01$ and ****$p < 0.0001$.

Compound-Taxus-containing serum for 12 h. Cell cycle changes of Smmc7721 and Bel7402 cells caused by Compound-Taxus-containing serum treatment were shown in Fig. 2(a). Significant accumulation in G2/M phase was found in a concentration-dependent manner in both Smmc7721 (Fig. 2(b), (e)) and Bel7402 cells (Fig. 2(c), (d)).

3.3. Compound Taxus promoted apoptosis of Smmc7721 and Bel7402 cells

To investigate the effect of compound Taxus on cell apoptosis, Smmc7721 and Bel7402 cells were treated with 0%, 5% or 10% Compound-Taxus-containing serum for 24 h. Representative results were shown in Fig. 3(a) and (b). After 0%, 5% or 10% Compound-Taxus-containing serum treatment, the apoptotic rates were (2.905 ± 1.779)%, (16.558 ± 6.705)% and (40.026 ± 8.221)% in Smmc7721, and (3.177 ± 1.869)%, (15.369 ± 6.679)% and (41.317 ± 7.890)% in Bel7402 cells, respectively, indicating that Compound-Taxus-containing serum promoted apoptosis in a concentration-dependent manner. To further explore the potential molecules involved, mitochondrial apoptosis-related proteins were detected using western blotting analysis. As was shown in Fig. 3(c) and (d), the proapoptotic effector Bax protein level was increased, whereas the antiapoptotic effector Bcl-2 protein level was decreased in a concentration-dependent manner. Consistent with the results of in vitro experiments, immunohistochemical staining of Bcl-2 and Bax showed that Compound Taxus inhibited Bcl-2 expression while increased Bax expression in Bel7402 tumor-bearing mice (Fig. 3(e) and (f)).

3.4. Compound Taxus suppressed the migration and invasion of Smmc7721 and Bel7402 cells

Considering that metastasis of tumor cells plays an important role in tumor progression, we performed wound healing and Transwell assay to evaluate the influences of Compound Taxus on the migration and invasion activities of HCC cells. As shown in Fig. 4 (a–d), Compound-Taxus-containing serum drastically retarded wound closure of Smmc7721 and Bel7402 cells in a concentration-dependent manner. Similarly, result of the Transwell assay showed that the number of migrated cells was significantly decreased by Compound Taxus treatment. The above results indicated that Compound Taxus apparently inhibited the migratory capacity of HCC cells. Furthermore, the Transwell invasion assay demonstrated that Compound-Taxus-containing serum treatment drastically

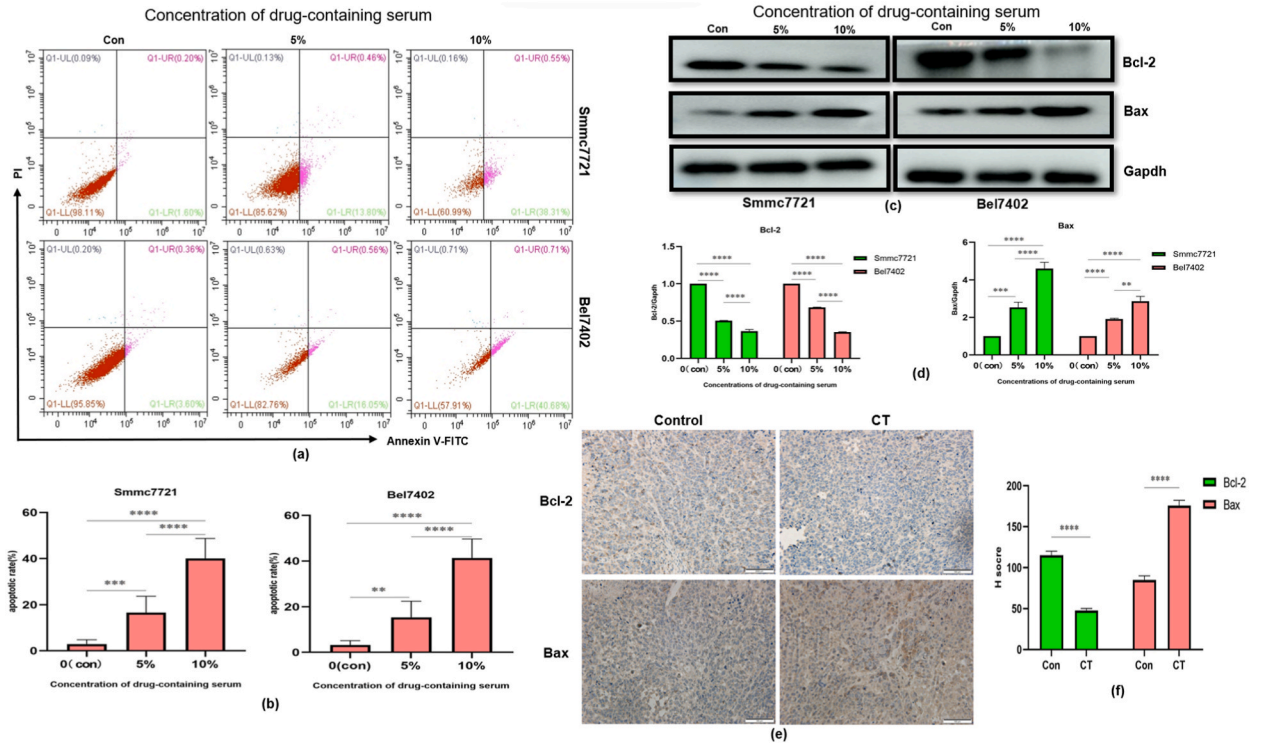

Fig. 3. Compound Taxus promoted apoptosis of Smmc7721 and Bel7402 cells. Smmc7721 and Bel7402 cells were treated with 0%, 5% or 10% Compound-Taxus-containing serum for 24 h and analyzed by flow cytometry. (a) Representative results of cell apoptosis from three independent experiments. (b) The apoptotic rates after Compound-Taxus-containing serum treatment in Smmc7721 and Bel7402 cells. (c,d) Expression levels of apoptosis-related proteins Bcl-2 and Bax were detected by western blotting, and the unadjusted images were provided in supplementary material. (e) Representative immunohistochemical (IHC) staining of Bcl-2 and Bax between the control group and the CT group in Bel7402 tumor-bearing mice. (f) IHC staining score for Bcl-2 and Bax protein. Values are represented (all data are expressed) as the mean ± SD. The experiment was repeated at least three times. $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$.

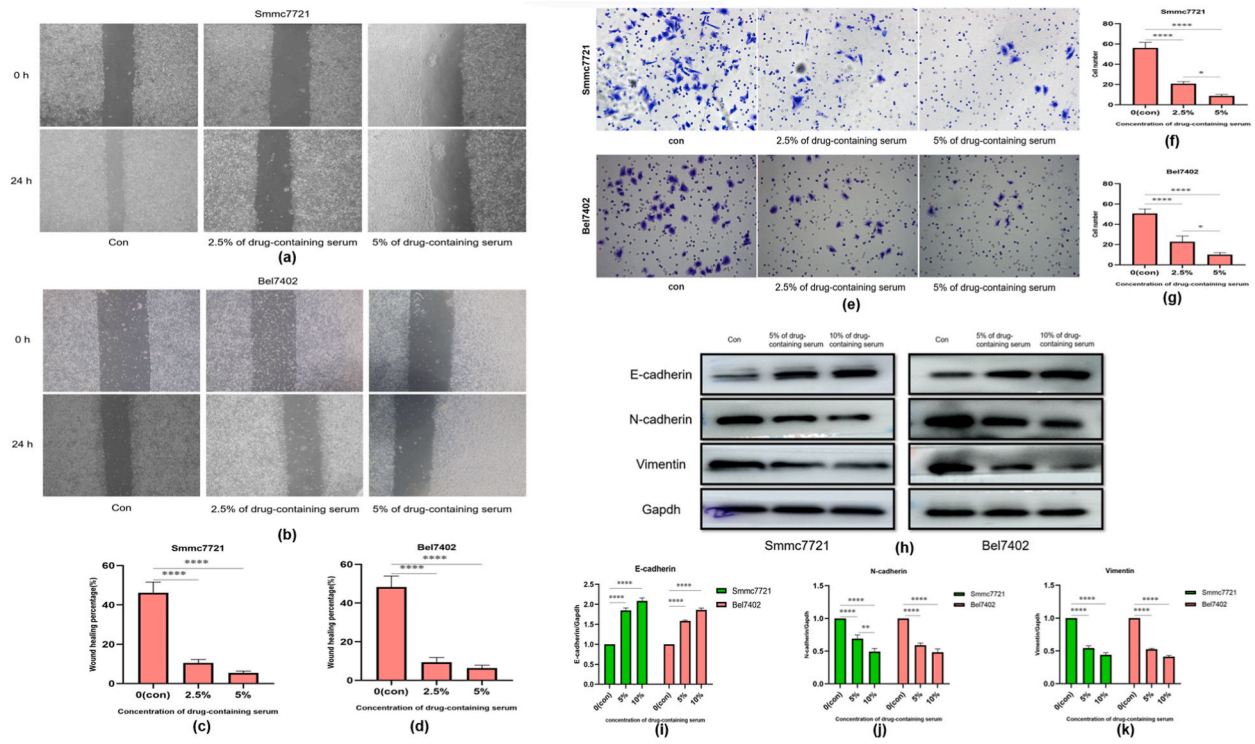

Fig. 4. Compound-Taxus-containing serum inhibited migration and invasion of Smmc7721 and Bel7402 cells. Smmc7721 and Bel7402 cells were treated with the indicated concentrations of Compound-Taxus-containing serum for 48 h. (a,b)Wound-healing assays were performed to evaluate the migration capacity. (c,d)The results of wound-healing assays were analyzed using ImageJ. (e) Transwell assays with matrigel were performed to evaluate the migration and invasion capacity. (f,g) The results of Transwell assays were analyzed using ImageJ. (h–k) Expression levels of EMT-related biomarkers were detected by western blotting, and the unadjusted images were provided in supplementary material. Values are represented (all data are expressed) as the mean ± SD. The experiment was repeated at least three times. Statistical significance was determined using two-tailed Student's t-test or one-way ANOVA. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

decreased the number of cells that invaded into the lower chamber through extracellular matrix (ECM) gels, suggesting that Compound Taxus restricted the invasive capacity of HCC cells (Fig. 4(e–g)). To further investigate the possible mechanisms involved, we detected the expression levels of select biomarkers of the epithelial-mesenchymal transition (EMT) process, which has been verified to engage in metastasis of tumor cells. Fig. 4(h–k) showed that Compound Taxus treatment increased E-cadherin expression while reduced the levels of N-cadherin and vimentin in both cell lines, demonstrating that Compound Taxus might exert its anti-metastasis effect partly by repressing the EMT process.

3.5. Compound Taxus inhibited VM formation and angiogenesis of Smmc7721 and Bel7402 cells

HUVEC, Smmc7721 and Bel7402 cells efficiently formed vasculogenesis-like networks on Matrigel within 24 h, and Compound-Taxus-containing serum inhibited the formation of such tubular structures in a concentration-dependent manner (Fig. 5(a–c, e)). In addition, the effect was further verified by quantification of master junctions and relative total segment length (Fig. 5(b–d, f)), indicating that Compound Taxus inhibited VM formation and angiogenesis of HCC cells. We further explored the possible molecule mechanisms. CD31 and VEGF are reported to be crucial regulators of angiogenesis [27], and VEGFA plays an important role in the formation of VM channels [28]. Immunohistochemical staining showed that Compound Taxus inhibited CD31 and VEGFA expression in Bel7402 tumor-bearing mice (Fig. 5(g–j)). In addition, MMP9 is a well-known biomarker of VM in tumor cell lines. Notably, it was found to be significantly downregulated by Compound Taxus treatment in Smmc7721 and Bel7402 cells (Fig. 5(k,l)).

3.6. Compound Taxus pretreatment inhibited irradiation-induced DNA damage repair in Smmc7721 and Bel7402 cells

To investigate the effect of Compound Taxus on irradiation-induced DNA damage repair, Smmc7721 and Bel7402 cells were pretreated with 0 or 5% Compound-Taxus-containing serum and then received 4Gy X-ray irradiation. DNA damage-induced Rad51 foci formation represents the status of the DNA repair system. Compared with the group of irradiation alone, pretreatment with 5% Compound-Taxus-containing serum significantly inhibited the formation of Rad51 foci in both Smmc7721 and Bel7402 cells (Fig. 6).

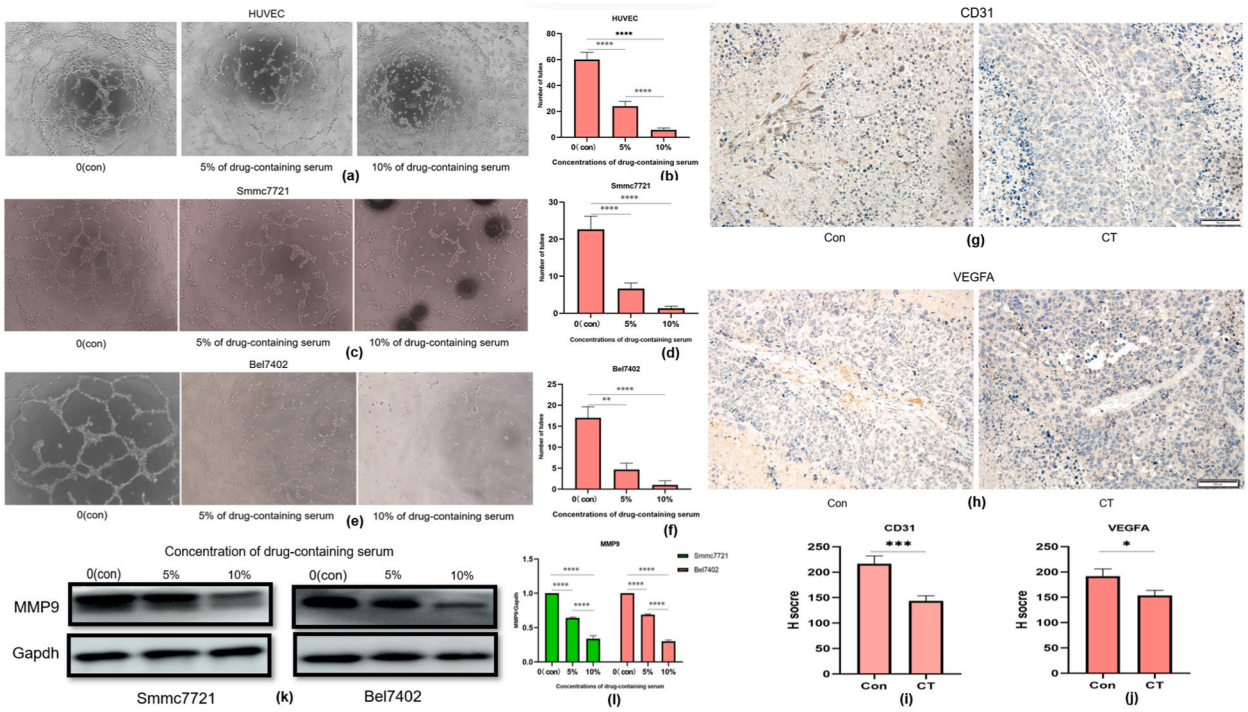

Fig. 5. Compound Taxus impaired angiogenesis of HUVEC and VM formation of HCC cells. (a–f) Different concentrations of Compound Taxus reduced tube formation by HUVEC, Smmc7721 and Bel7402 cells on Matrigel. The average number of master junctions and total segment length in five different fields were analyzed. (g,h) Representative IHC staining of CD31 and VEGFA between the control group and the CT group in Bel7402 tumor-bearing mice. (i,j) IHC staining score for CD31 and VEGFA protein. (k,l) Western blotting showed that MMP9 levels were substantially decreased following Compound Taxus treatment. The unadjusted images were provided in supplementary material. Values are represented (all data are expressed) as the mean ± SD. The experiment was repeated at least three times. *P < 0.05, **P < 0.01, ***P < 0.001.

3.7. Compound Taxus enhanced the radiosensitivity of Smmc7721 and Bel7402 cells

To investigate whether Compound Taxus enhances the cellular sensitivity to X-rays, Smmc7721 and Bel7402 cells were pretreated with 0 or 5% Compound-Taxus-containing serum for 12 h before X-ray irradiation (0–10 Gy). Radiation survival curves (Fig. 7(b) and (c)) were obtained from the colony formation results (Fig. 7(a)). The sensitizer enhancement ratio (SER) is a useful index for specifying intrinsic radiosensitivity of biological cell systems and is favoured by ICRU Report No. 30 [29]. It was defined as the ratio of the radiation dosage necessary to decrease the survival fraction (SF) to 0.5 in the absence of sensitizer to the dosage required to achieve the same SF with sensitizer. SER value higher than 1.20 indicates radiosensitization [30]. As was shown in Table 1 and Table 2, SF values at 2 Gy (SF2) of the RT + CT group were evidently lower than SF2 of the RT group in both Smmc7721 and Bel7402 cells. Compound-Taxus-containing serum enhanced the radiosensitivity of Smmc7721 and Bel7402 cells with SER of 1.46 and 1.59, respectively.

D0 is the mean lethal dose; Dq is the required threshold for cell damage; SF2 is the survival fraction at 2 Gy; SER is the radiation enhancement ratio.

3.8. Compound Taxus enhanced the therapeutic efficacy of irradiation in Bel7402 tumor-bearing mice

To further examine whether Compound Taxus can exert the radiosensitization effect in vivo, a Bel7402 tumor-bearing mouse model was established. Nude mice with subcutaneous Bel7402 tumors were randomly divided into four groups and received different treatments. As was illustrated in Fig. 2, the best anti-tumor therapeutic efficacy in Bel7402 tumor-bearing mice was found in the Comb group, and tumors were significantly inhibited with a smaller mean volume and lighter mean weight than other groups. Compared with the Con group, both the CT group and the RT group showed greater tumor suppression with decreased tumor volume and reduced tumor weight. The RT group presented better tumor inhibition than the CT group (Fig. 8(a), (b) and (c)). Mean tumor growth tripling time (TGT3), mean tumor growth delay time (TGD), enhancement factor (EF), mean tumor weight and mean tumor suppressor rate were shown in Table 3. Mean tumor suppressor rate of the Comb group was 90%, higher than that of the RT group (75%). EF was 1.55, indicating that Compound Taxus had the radiosensitization effect in Bel7402 tumor-bearing mice.

[a] TGT3: tumor growth tripling time. [b] TGD: tumor growth delay time (tumor growth tripling time of the treated group minus tumor growth tripling time of the Con group). [c] EF: enhancement factor (tumor growth delay time of the Comb group/tumor growth delay time of the RT group). [d] tumor suppressor rate (1 - mean tumor weight of the treated group/mean tumor weight of the Con group) ×

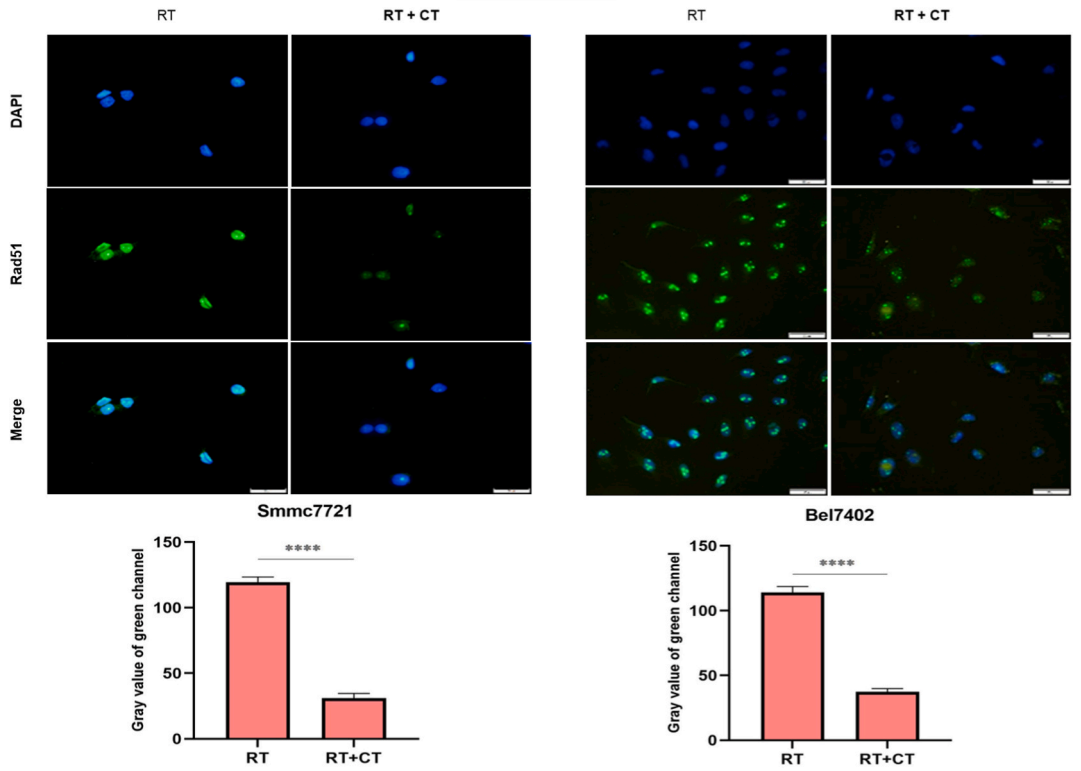

Fig. 6. Compound Taxus pretreatment inhibited the formation of Rad51 foci in Smmc7721 and Bel7402 cells. Smmc7721 and Bel7402 cells were pretreated with 0 or 5% Compound-Taxus-containing serum and then received 4Gy X-ray irradiation. Rad51 was analyzed for expression and distribution by immunofluorescence staining (Rad51(green), nuclear staining with DAPI (blue)). Scale bars represent 50 μm. Quantitative analysis of Rad51 foci formation was performed using gray value of green channel. Values are represented (all data are expressed) as the mean ± SD. The experiment was repeated at least three times. ****$p < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

100%.

3.9. Compound Taxus might exert its proapoptotic effect partly through downregulating the NF-κB signaling pathway

Evidence is accumulating to indicate that NF-κB activation may be an important feature of HCC [31–33]. To further explore the potential mechanisms involved in the above-mentioned biological activities of Compound Taxus, we examined whether Compound Taxus had an effect on the NF-κB signaling pathway. Smmc7721 and Bel7402 cells were treated with 0, 5% or 10% Compound-Taxus-containing serum for 24 h. Immunofluorescence staining results showed that protein expression and nuclear binding levels of NF-κB p65 were dramatically decreased by Compound-Taxus-containing serum treatment in Smmc7721 and Bel7402 cells (Fig. 9(a–b), Fig. 9(c–d)). In addition, western blotting analysis indicated that NF-κB p65 and p–NF–κB p65 protein levels were significantly inhibited by Compound-Taxus-containing serum in a concentration-dependent manner in Smmc7721 and Bel7402 cells (Fig. 9e–f). Moreover, we analyzed NF-κB p65 expression levels in tumor tissues by immunohistochemical staining and found that the amount of NF-κB p65 expression in the CT group was remarkably less than that in the control group (Fig. 9g–h).

To determine whether downregulation of the NF-κB pathway by Compound Taxus contributed to its proapoptotic effect on HCC cells, plasmid-mediated NF-κB p65 overexpression was used. After 48 h of pcDNA3.1-p65 or the empty vector transfection, Smmc7721 and Bel7402 cells were pretreated with 0 or 5% Compound-Taxus-containing serum for 12 h before X-ray irradiation (4Gy). As was shown in Fig. 10((a) and (b)), NF-κB p65 was successfully overexpressed. Compared with the RT group, Compound Taxus pretreatment significantly increased the apoptotic rate of both Smmc7721 and Bel7402 cells, while NF-κB p65 overexpression reversed the pro-apoptotic effect of Compound Taxus (Fig. 10(c), (d) and 10(e)).

4. Discussion

HCC is one of the most common malignant tumors around the world with the highest morbidity and mortality rates. The five-year survival rate of HCC is only 20% due to factors including late diagnosis, rapid progression, poor response to treatments in the advanced stage, and high recurrence rate. With the continuous advancements of technology, RT has been widely used as an integral part of

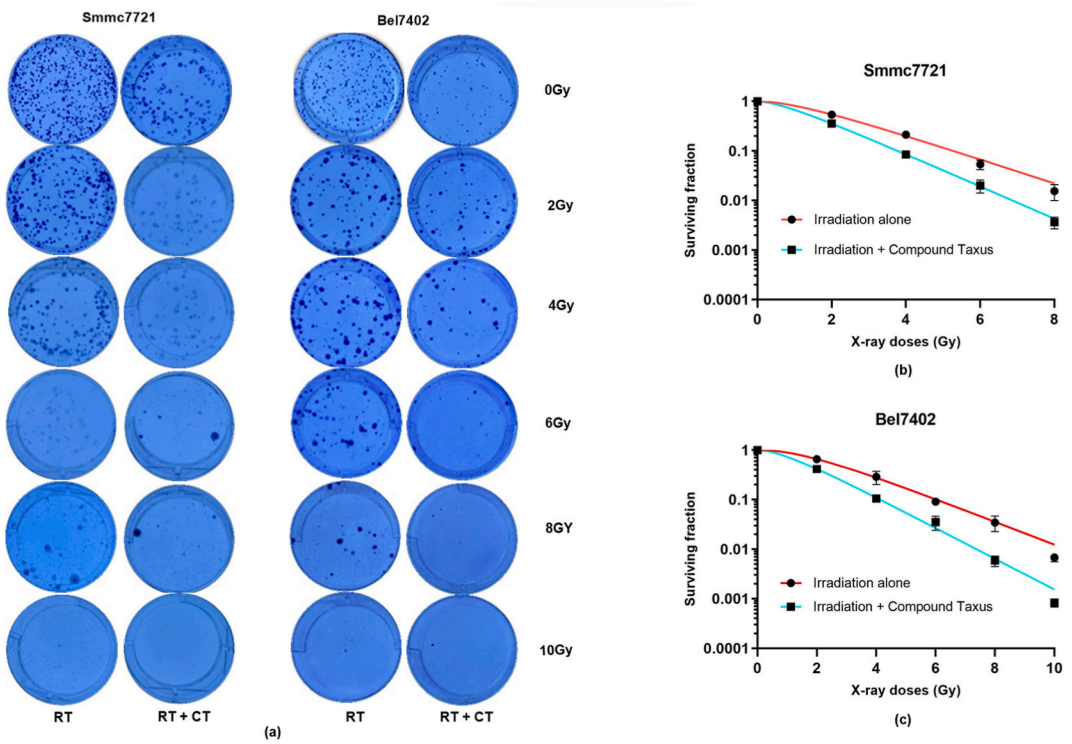

Fig. 7. Compound Taxus enhanced the radiosensitivity of Smmc7721 and Bel7402 cells. Smmc7721 and Bel7402 cells were pretreated with 0 or 5% Compound-Taxus-containing serum for 12 h and then exposed to X-ray irradiation (0–10 Gy). (a) Representative graphs of colony formation of Smmc7721 and Bel7402 cells. (b、c) Radiation survival curves of Smmc7721 and Bel7402 cells. Values are represented (all dates are expressed) as the mean ± SD. The experiment was repeated at least three times. RT: radiotherapy; CT: Compound Taxus.

Table 1
Multiple target model parameters of S.mmc7721

Group	D0(Gy)	Dq (Gy)	SF_2	SER(SF = 0.5)
RT	1.790 ± 0.221	1.298 ± 0.538	0.537 ± 0.036	1.46
RT + CT	1.351 ± 0.282	0.915 ± 0.709	0.363 ± 0.004	
p	<0.05	<0.05	<0.05	

Table 2
Multiple target model parameters of Bel7402.

Group	D0(Gy)	Dq (Gy)	SF_2	SER(SF = 0.5)
RT	1.912 ± 0.335	1.913 ± 0.927	0.634 ± 0.024	1.59
RT + CT	1.415 ± 0.253	1.071 ± 0.650	0.383 ± 0.007	
p	<0.05	<0.05	<0.05	

D0 is the mean lethal dose; Dq is the required threshold for cell damage; SF2 is the survival fraction at 2 Gy; SER is the radiation enhancement ratio.

comprehensive treatment for HCC. However, limited radiosensitivity of tumour poses great challenges to expectant treatment outcomes. It was reported that the radiosensitivity of 16 HCC patient-derived cell lines was mainly identified as moderately sensitive and radioresistant, with less than 20% classified as sensitive [34]. Therefore, it is of great significance to enhance the sensitivity of HCC to RT. Nevertheless, little information about radiosensitizers of HCC is available. In the present study, we demonstrated that Compound Taxus exerted marked antitumor activity with its anti-proliferative, pro-apoptotic, and anti-metastatic effects on HCC cells. Furthermore, we also found for the first time to our knowledge that Compound Taxus significantly enhanced the radiosensitivity of HCC cells in vitro and in vivo. RT in combination with Compound Taxus showed the best anti-tumor therapeutic efficacy in Bel7402 tumor-bearing mice.

Compound Taxus capsule has been increasingly prescribed as an auxiliary modality to chemotherapy for NSCLC management. A meta-analysis demonstrated the synergistic effect of chemotherapy in combination with Compound Taxus capsule on clinical outcomes of NSCLC patients. The findings showed that the combination therapy significantly improved clinical efficacy, quality of life and

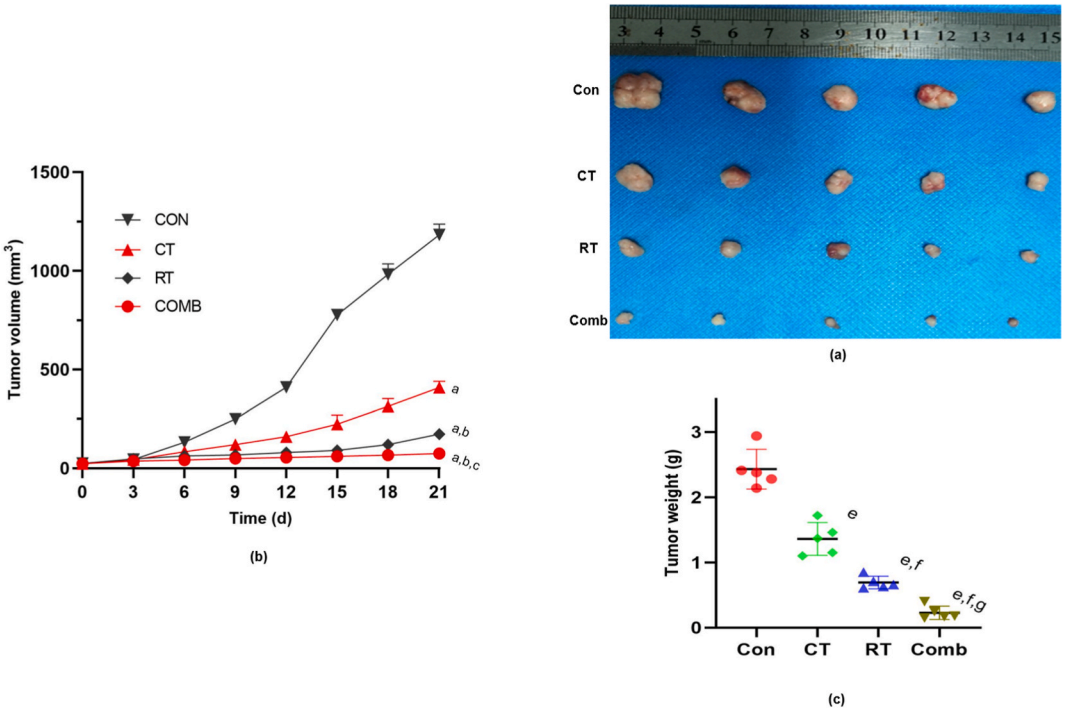

Fig. 8. Compound Taxus enhanced the therapeutic efficacy of irradiation in Bel7402 tumor-bearing mice. (a) Photographs of the isolated tumors in different groups. (b) Bel7402 tumor growth curves in different groups. Values are represented (all data are expressed) as the mean ± SD. Significant differences in tumor volume were found between Comb, RT, CT and Con groups (a, $p < 0.0001$ compared with the Con group; b, $p < 0.0001$ compared with the CT group; c, $p < 0.01$ compared with the RT group). (c) The weights of stripped tumors in different groups. (e, $p < 0.0001$ compared with the Con group; f, $p < 0.0001$ compared with the CT group; g, $p < 0.01$ compared with the RT group).

Table 3
Tumor growth inhibition in Bel7402 tumor-bearing mice receiving different treatments.

Group	Numbers of mice	Mean TGT3 (day)[a]	Mean TGD (day)[b]	EF[c]	Mean tumor weight (g)	Mean tumor suppressor rate (%)[d]
Con	5	3.5	–	–	2.4	–
RT	5	10	6.5	–	0.6	75
Comb	5	13.6	10.1	1.55	0.2	90

decreased the possibility of adverse reactions [34]. Taxus is an invaluable woody species with important medical value and its extracts are composed of many chemical compounds, such as phenols, flavonoids and terpenes [11]. Taxol, as a unique secondary metabolite from the bark of Taxus, was found to be a natural antitumor medicine, which was widely applied in the treatment of breast, ovarian and lung cancers [35,36]. Hafezi K et al. [37] found that a polyphenolic compound named α-Conidendrin from Taxus yunnanensis played a significant antiproliferative role in breast cancer cells by inducing cell cycle arrest and apoptosis. Qu C et al. [38] demonstrated that water decoction from Taxus cuspidate possessed the inhibitory effect on pancreatic tumor growth. Jiang YQ et al. [39] showed that Taxus chinensis var. Could reverse cisplatin resistance in NSCLC stem cells. The capacities to inhibit proliferation and promote apoptosis of tumor cells are considered to be essential criteria for developing new antitumor drugs. Our results displayed that Compound Taxus significantly decreased viability, induced G2/M arrest, promoted apoptosis, suppressed migration and invasion, and inhibited VM formation and angiogenesis in Smmc7721 and Bel7402 cells, providing clear evidence that Compound Taxus possesses marked antitumor activity with its anti-proliferative, pro-apoptotic, and anti-metastatic effects on HCC cells. In addition, we found that Compound Taxus inhibited irradiation-induced DNA damage repair in Smmc7721 and Bel7402 cells, and we obtained the radiation survival curves from the colony formation results and demonstrated that Compound Taxus enhanced the radiosensitivity of Smmc7721 and Bel7402 with SER of 1.347 and 1.360, respectively. Furthermore, we applied the combination of RT and Compound Taxus to Bel7402 tumor-bearing mice and observed the most significant tumor control compared with that of RT alone or Compound Taxus alone. Combination of RT and Compound Taxus repressed tumor volume and tumor weight more effectively. In brief, we identified that Compound Taxus sensitized HCC cells to radiation in xenograft mouse model consistent with in vitro results, highlighting its potential to become a promising radiosensitizer of HCC in the future.

Relative radiosensitivity of cells is reported to be determined by cell cycle phase. The G2-M phase is most sensitive to irradiation, while the G1 phase is less sensitive and the latter part of the S phase is least sensitive. The idea of synchronizing tumor cells in a specific

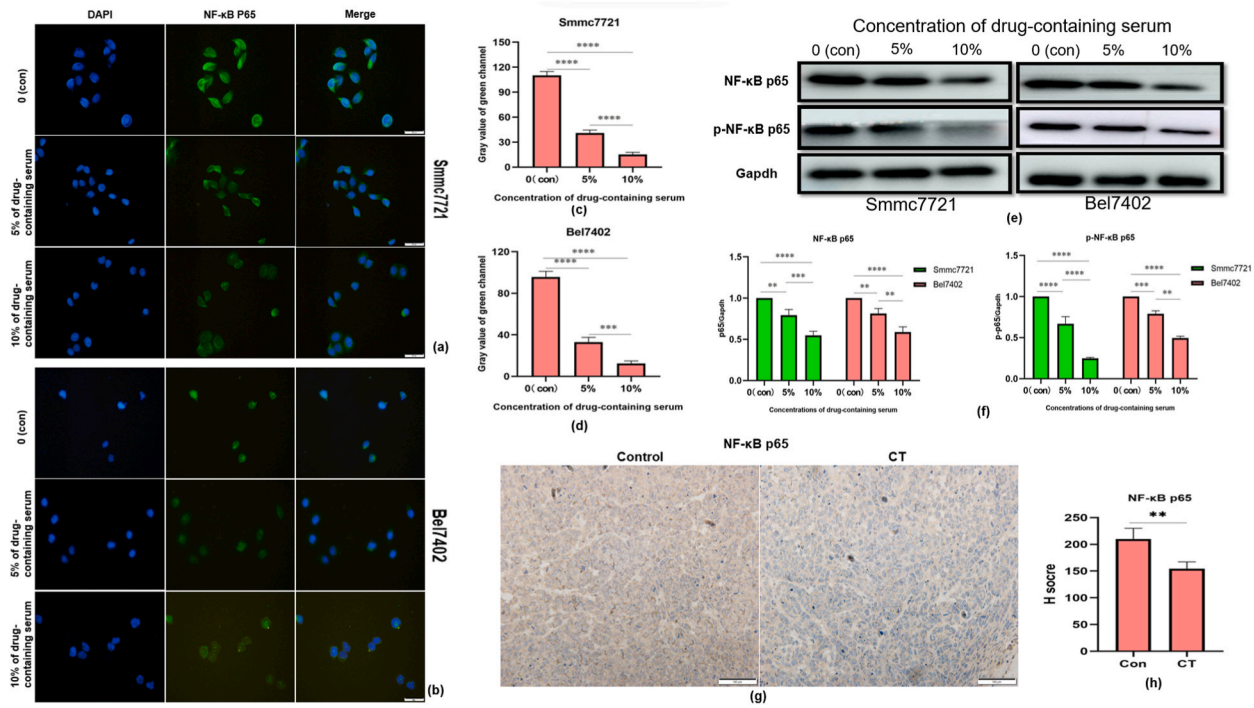

Fig. 9. Compound Taxus induced inhibition of the NF-κB signaling pathway. Smmc7721 and Bel7402 cells were treated with 0, 5% or 10% Compound-Taxus-containing serum for 24 h. (a,b) NF-κB p65 expression and distribution were analyzed by immunofluorescence staining. (c,d) Quantitative analysis of NF-κB p65 was performed using gray value of green channel. (e,f) The protein expression levels of NF-κB p65 and p–NF–κB p65 were evaluated by western blotting and the unadjusted images were provided in supplementary material. (g) Representative IHC staining of NF-κB p65 between the control group and the CT group in Bel7402 tumor-bearing mice. (h) IHC staining score for p65 protein. Values are represented (all data are expressed) as the mean ± SD. The experiment was repeated at least three times. ***P < 0.001, ****P < 0.0001. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

cell cycle phase sensitive to radiation was viewed as a potentially valuable way to enhance the therapeutic efficacy of RT [40]. Stingl L et al. reported that novel HSP90 inhibitors exerted the radiosensitization effect on tumor cells by induction of G2/M arrest and depletion of S phase, DNA damage and repair protraction [41]. It was also reported that genistein radiosensitized breast cancer cells through G_2/M cell cycle arrest and mitochondria-mediated apoptosis [25]. In the present study, we found that Compound Taxus induced significant accumulation at G2/M phase in Smmc7721 and Bel7402 cells in a concentration-dependent manner, which might contribute to the radiosensitization effect of Compound Taxus.

DNA is the critical target to achieve RT-induced cell death. The theory of radiobiology suggested that the primary biological task of cell cycle arrest is to spare sufficient time for DNA repair. Once cells can not repair the serious DNA damages, they will ultimately enter the death process [42,43]. DNA repair systems are the main mechanisms for radiotherapeutic resistance. DNA damage-induced Rad51 foci are considered to reflect homologous recombination (HR) repair of DNA double-strand breaks and represent the level of HR functionality [25]. Our results showed that Compound Taxus pretreatment markedly inhibited DNA damage-induced Rad51 foci formation in Smmc7721 and Bel7402 cells, indicating that disturbance of DNA HR repair by Compound Taxus might be the major cause leading to impairment of DNA repair in cells at G2/M phase after RT.

It was reported that NF-κB overactivation played a key role in radioresistance in various types of cancer, and its inhibition could diminish the resistance [24,44–46]. NF-κB is a critical transcription factor that plays essential roles in diverse biological and pathological processes, such as cell cycle progression, apoptosis, oxidative stress responses, invasion, and metastasis [47]. All these properties, induced by NF-κB activation, are linked to enhanced radioresistance. Taking the complexity of effects conferred by NF-κB activation in cancer cells into overall consideration, a conclusion can be made that this transcription factor has a crucial role in determining the therapeutic effect of RT [48]. Therefore, RT combined with NF-κB pathway inhibitors is expected to achieve an increase in radiosensitivity. Several in vitro and in vivo studies reported that Curcumin, a well-known inhibitor of NF-κB, exerted the radiosensitization effect in different types of tumors, such as oral squamous cell carcinoma, burkitt's lymphoma and HCC, via inhibiting radiation-induced NF-κB activation [49–51]. In this study, we observed that Compound Taxus downregulated the protein expression levels of NF-κB p65, p–NF–κB p65 and the antiapoptotic effector Bcl-2, while upregulated the proapoptotic effector Bax expression in Smmc7721 and Bel7402 cells. Consistent with in vitro observations, Compound Taxus likewise influenced the expression of NF-κB p65, Bcl-2 and Bax in the xenograft tumor tissue, as displayed by our immunohistochemical staining results. In addition, NF-κB p65 overexpression reversed the proapoptotic effect of Compound Taxus. These findings demonstrate that Compound Taxus might exert its proapoptotic effect, at least in part, through downregulating the NF-κB signaling pathway.

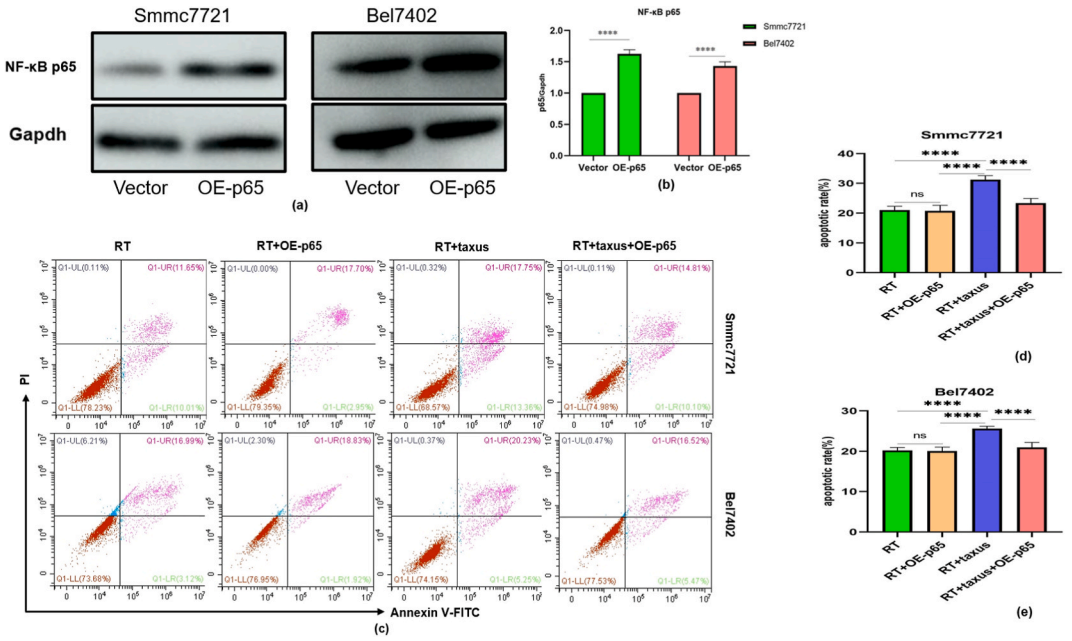

Fig. 10. NF-κB p65 overexpression reversed the proapoptotic effect of Compound Taxus. Smmc7721 and Bel7402 cells were transfected with pcDNA3.1-p65 or the empty vector. (a,b) Western blotting was performed to confirm NF-κB p65 overexpression after transfection and the unadjusted images were provided in supplementary material. (c,d,e) After 48 h of transfection, cells were pretreated with 0 or 5% Compound-Taxus-containing serum for 12 h before X-ray irradiation (4Gy) and then analyzed by flow cytometry. (c) Representative results of cell apoptosis from three independent experiments. (d,e) The apoptotic rates of the four groups in Smmc7721 and Bel7402 cells. Values are represented (all data are expressed) as the mean ± SD. The experiment was repeated at least three times. ****$P < 0.0001$.

There are several limitations in the present research. First, all in vitro experiments about anti-tumor activities and radiosensitization effect of Compound Taxus on HCC cells were performed only using two well-established HCC Cell lines-Smmc7721 and Bel7402. It will be of better and stronger clinical significance if patient-derived HCC cells of primary culture can be established and used. Second, in vivo experiments were performed using only one Bel7402 tumor-bearing mouse model. However, to strengthen the translational relevance of the findings, other relevant in vivo models (e.g., patient-derived xenografts or orthotopic models) should be considered in future research. Third, further studies are needed to understand the detailed molecular mechanisms involved in the antitumor activity and radiosensitization effect of Compound Taxus on HCC cells.

5. Conclusions

Our study not only suggests that Compound Taxus has anti-proliferative, pro-apoptotic, and anti-metastatic effects on HCC cells, but also provides evidence that Compound Taxus may sensitize HCC cells to RT partly by inducing G2/M arrest, inhibiting irradiation-induced DNA damage repair and ultimately promoting irreversible apoptosis, which makes it possible for Compound Taxus to become a promising auxiliary modality for HCC management and a potential radiosensitizer of HCC in the future.

Ethics statement

All the animal experiments were approved by the Medical Ethics Committee of Shandong Provincial Hospital(No. 2020-021) and performed in accordance with the relevant guidelines and regulations.

Additional information

No additional information is available for this paper.

Data availability statement

The data used to support the findings of this study are available from the corresponding authors upon reasonable request.

CRediT authorship contribution statement

Hui-quan Gao: Writing – review & editing. **Xiang-mao Bu:** Writing – review & editing. **Wei Jiang:** Writing – review & editing, Conceptualization. **Yan-zhen Wan:** Writing – review & editing. **Wei Song:** Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e27345.

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