

ORIGINAL ARTICLE

Tanshindiol-C suppresses *in vitro* and *in vivo* hepatocellular cancer cell growth by inducing mitochondrial-mediated apoptosis, cell cycle arrest, inhibition of angiogenesis and modulation of key tumor-suppressive miRNAs

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Summary

Purpose: Hepatocellular carcinoma causes considerable mortality and no efficient chemotherapy is available. Novel molecules of plant origin may prove beneficial in the development of therapy of hepatocellular carcinoma. In this study we examined the anticancer effects of Tanshindiol-C (TC) against the hepatocellular carcinoma SNU-4235 cell line.

Methods: Proliferation rate of the SNU-2435 cells was determined by MTT assay. Apoptosis was confirmed by DAPI and annexin V/PI staining. Cell cycle analysis was performed by flow cytometry. MicroRNA expression was checked by qRT-PCR and protein expression by western blotting. The *in vivo* evaluation of TC was performed in xenografted mice models.

Results: TC inhibited the growth of the SNU-4235 cells and exhibited an IC_{50} of 20 μ M. Investigation of the underlying mechanism revealed that TC triggered apoptotic death of the SNU-4235 cells which was also associated with enhancement

of the expression of Bax and decrease in the expression of Bcl-2. TC also caused arrest of the cells in the G2/M phase of the cell cycle and also exerted angiogenic effects. TC also enhanced the expression of the tumor suppressor microRNA-21, 222 and 31. *In vivo* evaluation of TC revealed that it could inhibit the tumor weight volume, suggestive of the anticancer potential of TC.

Conclusions: In brief, tanshindiol-C exerts anticancer effects on hepatocellular carcinoma by induction of apoptosis and cell cycle arrest, along with inhibiting the angiogenesis and the expression of tumor suppressive microRNAs. TC could also inhibit the growth of the xenografted tumors and hence could prove to be a potential anticancer agent.

Key words: angiogenesis, apoptosis, hepatocellular carcinoma, microRNA, tanshindiol-C

Introduction

Liver cancer is one of the most devastating malignancies and is unevenly distributed around the globe. Around 80% hepatocellular carcinomas (HCC) are reported from sub-Saharan Africa or Eastern Asia. Furthermore, it has been reported that about 50% of HCC carcinoma cases occur in

China [1]. HCC causes tremendous mortality and is currently ranked as the 5th most prevalent type of cancer across the globe. For proper management of HCC, early detection and availability of efficient chemotherapeutic drugs is imperative. Hence, the identification and screening of new molecules

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against HCC is considered very important for the development of efficient therapy for HCC [2]. Since synthetic drugs have associated side effects, natural products may prove having less unwanted effects and at least equal efficacy of the drugs currently in use in HCC [3]. Plants and microbes have provided a diversity of drugs for treatment of severe diseases and they are likely to continue to serve as resource of more important drugs [3]. Plants are specialised to produce metabolites to combat environmental stresses. Such metabolites, commonly referred to as secondary metabolites have been employed for the treatment of diseases such as cancer [4]. For example, paclitaxel and camptothecins are among the common anticancer agents of plant origin [5,6]. Although these secondary metabolites have been chemically classified into different groups, coumarins form an important group with tremendous pharmacological potential [7]. In the present study the anticancer effects of a plant derived compound Tanshindiol-C (TC) was examined against an HCC cell line. It was found that TC exhibits remarkable anticancer effects on the HCC cells. The anticancer effects were due to induction apoptotic cell death which was associated with upregulation of Bax and downregulation of Bcl-2. Furthermore, TC could also induce G2/M cell cycle arrest of HCC cells. We also investigated the effects of two tumor-suppressive microRNAs and found that TC caused increase in the expression of tumor-suppressive microRNAs at IC₅₀. In addition, evaluation of the TC *in vivo* revealed that it could inhibit the tumor volume and weight as well as angiogenesis, indicative of the anticancer potential of TC.

Methods

Cell lines and culturing conditions

The hepatocellular carcinoma cell line SNU-4235 was obtained from American Type Culture Collection. The cells were maintained in Dulbecco's modified Eagle's medium in CO₂ incubator (Thermo Scientific, Waltham, Massachusetts, USA) at 37°C with 98% humidity and 5% CO₂.

Proliferation assay

The anticancer effect of TC was assessed on HCC cell line by MTT assay as described previously by Mosmann [8]. The hepatocellular carcinoma cells were subjected to treatment with varied concentrations of TC and the proliferation rate was determined by absorbance at 570 nm.

Apoptosis assay

Effects of TC on the induction of apoptosis were determined by DAPI staining as described previously [9]. In brief, the HCC cells (0.6×10⁶) were grown in 6-well plates. Following 12 hrs of incubation, the cells were subjected to TC treatment for 24 hrs at 37°C. The cell cul-

tures were then centrifuged and the pellets were washed with PBS. Thereafter, the cells were DAPI stained, centrifuged and washed with phosphate buffered saline (PBS). Finally, the nuclear morphology of the stained cells was examined by fluorescence microscopy. The percentage of the apoptotic cells was estimated by annexin V/propiidium iodide (PI) staining as previously described [10].

Cell cycle analysis

The distribution of the HCC cells in different cycle phases was performed by flow cytometry after PI staining. In brief, the HCC cells were grown in 6-well plates and treated with TC for 24 hrs. The cells were then collected, PBS-washed, and fixed with ethanol (70%). After overnight incubation at 4 °C, the cells were subjected to PI staining and subjected to flow cytometry.

cDNA synthesis and quantitative RT-PCR

RNA was isolated from the HCC cells by Trizol reagent and then transcribed into cDNA using RevertAid cDNA synthesis kit. The expression of tumor suppressive microRNAs (microRNA-21, microRNA-222 and microRNA-31) was determined by qRT-PCR as described previously [11].

Western blotting

Following the lysis of the HCC cells in RIPA lysis buffer, the protein content of each lysate was estimated by the bicinchoninic acid (BCA) assay. The samples were loaded on the SDS-PAGE and the gels were transferred to nitrocellulose membranes and subjected to treatment with primary antibody at 4°C for 24 hrs. After this, the membranes were incubated with HRP-conjugated secondary antibody (1:1000) for 50 min at 25°C. Enhanced chemi-luminescence reagent was used to visualise the protein bands.

In vitro angiogenesis assay

The *in vitro* angiogenesis assay was carried out by using an Angiogenesis kit (Kurabo, Japan). In brief, human umbilical vein endothelial cells (HUVECs) and fibroblasts were co-cultured in 24-well plates and SNU-4235 were cultured in the upper chamber, separated from the lower chamber by a membrane. The upper chamber was removed after day 7 and HUVECs were stained with anti-CD31 antibodies. The formation of tubes was evaluated in 10 random fields as previously described.

In vivo study

The experiments were performed in accordance with National Institutes of Health standards for the care and use of laboratory animals and approved by the Institutional Animal Care and Committee of the Institute. In this study 4-week-old female immunodeficient nude mice were kept in the animal facility as per the National Institutes of Health standards for the care and use of laboratory animals. Mice were injected with 5×10⁶ SNU-4235 cells subcutaneously at the left flank. As the tumors became apparent (~4 mm after about 2 weeks), the mice (n=5 for each group) were injected intraperitoneally with DMSO (0.1%) dissolved TC and diluted

with 100 μ L normal saline at 20 mg/kg body weight. TC was administered to the mice three times a week, while the control mice were administered with DMSO (0.1%) in normal saline only. At the end of the study, the mice were sacrificed and tumors were harvested for assessment of tumor growth and other investigations.

Results

TC inhibits antiproliferative effects on the HCC

The anticancer activity of TC (Figure 1) was examined on SNU-4235 HCC cells by MTT assay. It was found that TC exerts antiproliferative effects on the HCC cell line with an IC_{50} of 20 μ M (Figure 2). In addition, it was found that the anticancer effects of TC on the HCC was dose-dependent.

TC triggers apoptosis in HCC cells

To decipher if TC triggers apoptotic cell death of the HCC, the cells were treated with TC and subjected to DAPI staining. The results of DAPI assay showed that TC induced apoptotic cell death in the HCC (Figure 3). The Annexin V/PI staining showed that the apoptotic cell populations increased with

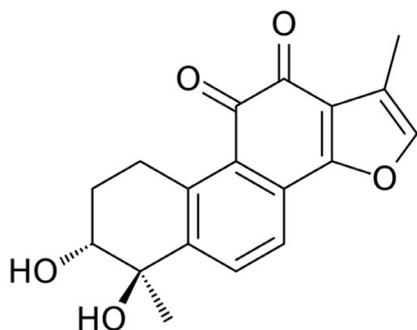


Figure 1. Chemical structure of Tanshindiol-C.

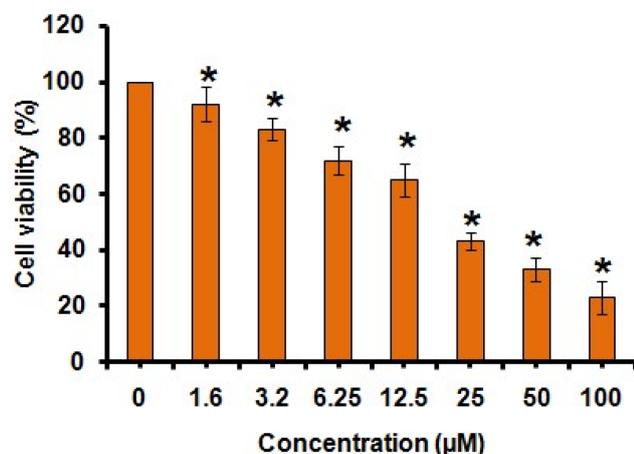


Figure 2. Effect of Tanshindiol-C on the viability of the hepatocellular carcinoma SNU-4235 cells. The experiments were performed in triplicate and expressed as mean \pm SD (* p < 0.01).

increase in the concentration of TC (Figure 4). Analysis of the protein expression of Bax and Bcl-2 showed that the expression of Bax increased which was associated with decrease in the expression of Bcl-2 (Figure 5).

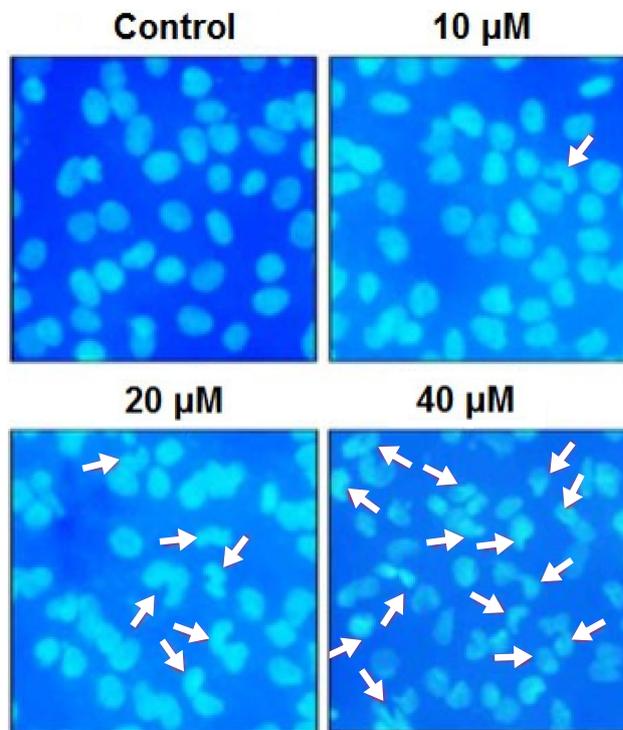


Figure 3. Tanshindiol-C triggers apoptotic cell death of the SNU-4235 cells as depicted by DAPI staining. The experiments were repeated in triplicate. Apoptosis was concentration-dependent and was highest at 40 μ M of Tanshindiol-C (arrows).

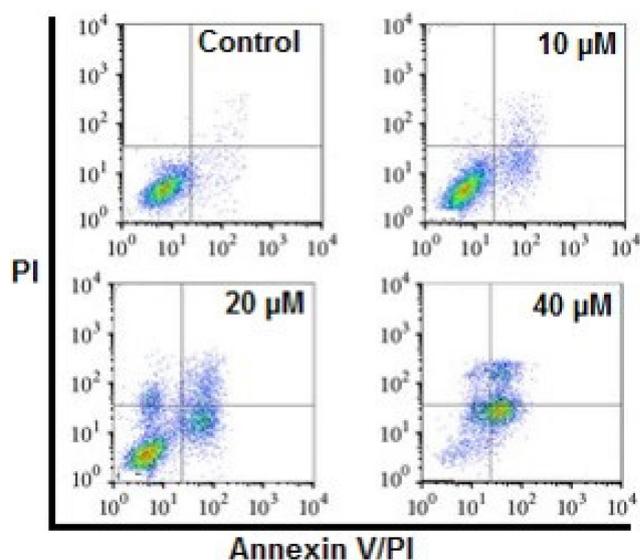


Figure 4. Determination of the percentage of the apoptotic cells by annexin V/PI staining. The experiments were repeated in triplicate and show that Tanshindiol-C triggers apoptotic cell death of SNU-4235 cells in a concentration-dependent manner.

TC causes G2/M arrest of HCC cells

The effects of TC on the distribution of SNU-4235 cells in various cell cycle phases was assessed by flow cytometry and it was found that TC caused

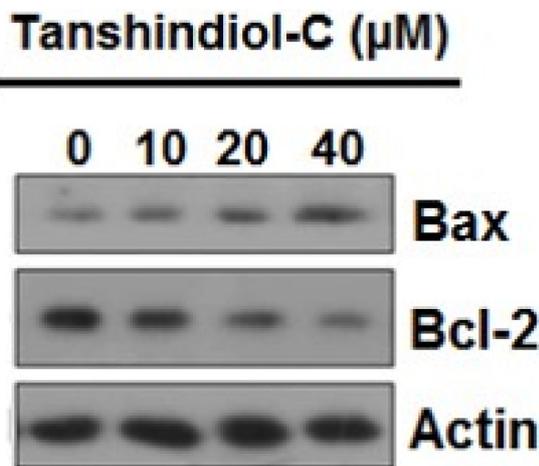


Figure 5. Effect of Tanshindiol-C on the expression of Bax and Bcl-2 in the Tanshindiol-C treated SNU-4235 cells. The experiments were repeated in triplicate and show that Tanshindiol-C upregulates the Bax and downregulates Bcl-2 expression concentration-dependently.

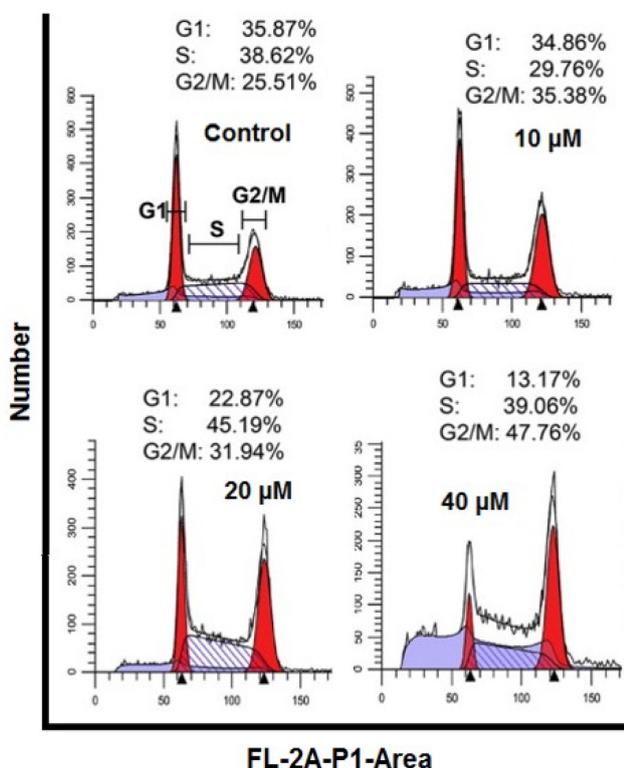


Figure 6. Tanshindiol-C triggers G2/M cell cycle arrest of the SNU-4235 cells in a concentration-dependent manner as depicted by flow cytometry. The experiments were performed in triplicate. Tanshindiol-C triggers G2/M cell cycle arrest of the SNU-4235 cells in a concentration-dependent manner as depicted by flow cytometry. The experiments were performed in triplicate.

remarkable increase in the percentage of the SNU-4235 cells in the G2 phase of the cell cycle. The percentage of SNU-4235 cells in the G2 phase increased from 25.51% to 47.76% upon treatment with TC (Figure 6). These results clearly indicate that TC induces G2/M cell cycle arrest of the HCC cells.

TC increases the expression of tumor suppressive microRNAs

Investigation of the expression of three tumor suppressive microRNAs (microRNA-21, microRNA-222 and microRNA-31) in TC-treated and untreated HCC cells showed that TC treatment enhanced the expression of all these microRNAs (Figure 7) indicating that TC might exert its anticancer effects at least in part via upregulation of suppressive microRNAs.

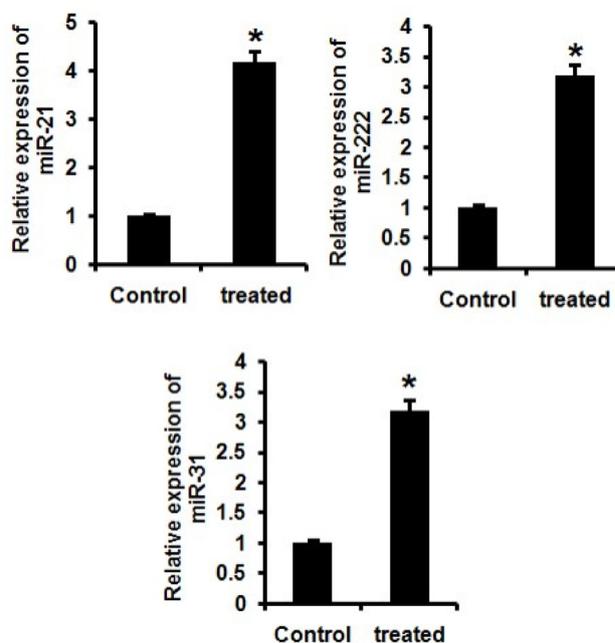


Figure 7. Effect of Tanshindiol-C on the expression of 3 tumor-suppressive microRNAs. The experiments were performed in triplicate and expressed as mean ± SD (*p < 0.01).

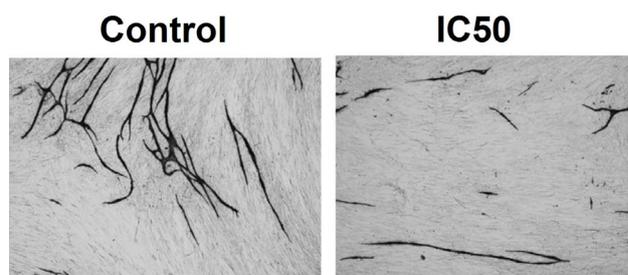


Figure 8. Effect of Tanshindiol-C on the angiogenesis of the cells as depicted by *in vitro* angiogenesis assay. The experiments were performed in triplicate and show that Tanshindiol-C inhibits angiogenesis of SNU-4235 cells.

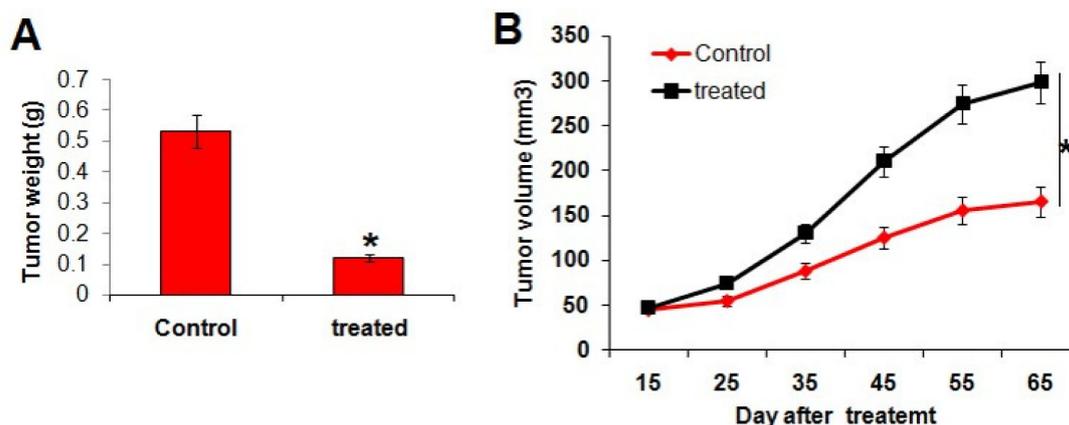


Figure 9. Effect of Tanshindiol-C on (A) Tumor weight (B) Tumor volume of the xenografted tumors. The experiments were performed in triplicate and expressed as mean \pm SD (* $p < 0.01$).

TC inhibits the angiogenesis of the HCC

The anti-angiogenesis potential of TC was investigated by Angiogenesis kit. The results showed that TC inhibited the tube formation of SNU-4235 cells, indicative of the anti-angiogenetic effects of TC (Figure 8).

TC inhibits tumor growth in vivo

TC exhibited considerable anticancer effects *in vitro* and therefore, we investigated its anticancer effects *in vivo*. The results revealed that TC inhibited the growth of the xenografted tumors at 20 mg/kg. Additionally, TC could also decrease the weight and volume of the xenografted tumors (Figure 9A and B).

Discussion

HCC is one of the devastating types of cancers, responsible for considerable mortality across the globe [12]. The treatment of HCC is ineffective due to its late diagnosis and the lack of viable treatment of options. Besides, the existing anticancer agents have adverse effects, negatively affecting the patient quality of life [13]. In this study, the anticancer effects of a plant-derived molecule Tanshindiol-C (TC) were investigated against the HCC SNU-4235 cell line. TC inhibited the growth of the SNU-4235 cells concentration dependently as depicted by the MTT assay. The anticancer effects of TC were due to the induction of apoptotic cell death which was confirmed by DAPI staining. Annexin V/PI staining further showed that the apoptotic cell populations increase with increased in the concentration of TC which was also associated with increase in the Bax/Bcl-2 ratio. Apoptosis is one of the important cellular processes which leads to exclusion of defective cells from the body [14]. Previous investigations have shown that many plant-derived molecules

induce apoptotic cell death of cancer cells [15]. In addition to apoptosis, anticancer drugs also inhibit the growth of cancer cells by causing arrest of the cells at different cell cycle checkpoints [16]. In this study we found that TC arrests the SNU-4235 cells at the G2/M checkpoint, preventing them to complete the cell cycle. MicroRNAs are small RNA molecules which do not code for proteins synthesis. These microRNAs regulate several cellular processes such as apoptosis [17]. Some of these microRNAs promote the growth of cancer cells, while others act as tumor suppressors. In this study we investigated the effect of TC on the expression of tumor suppressor microRNA-21, 222 and 31 in TC-treated and untreated SNU-4235 cells and found that the expression of all these tumor suppressive microRNA increased upon TC treatment. Furthermore, angiogenesis is an important process in tumorigenesis and subsequent metastasis of cancer [18] and the results of *in vitro* angiogenesis assays revealed that TC prevents angiogenesis of SNU-4235 cells. Given the promising results, the anticancer effects of TC were also evaluated against the xenografted tumors and it was found that TC reduced the weight and volume of the xenografted tumors, indicative of its anticancer potential.

Conclusion

In conclusion, TC exerts anticancer effects on the HCC cells by inducing apoptosis and cell cycle arrest. It also inhibits the angiogenesis and the expression of the tumor suppressive microRNAs. TC could also inhibit the growth of the xenografted tumors and hence deserves further investigations.

Conflict of interests

The authors declare no conflict of interests.

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