TTF1, in the Form of Nanoparticles, Inhibits Angiogenesis, Cell Migration and Cell Invasion in Vitro and In Vivo in Human Hepatoma through STAT3 Regulation

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Abstract: TTF1-NP(5,2′,4′-trihydroxy-6,7,5′-trimethoxyflavone nanoparticles), derived from the traditional Changbai Mountain medicinal plant Sorbaria sorbifolia (SS), has been showed its anti-cancer effect in various liver cancer cell types and tissues. The present study was designed to evaluate the antitumor mechanism of the TTF1-NP against HepG2 hepatoma cells and HepG2 cells-induced hepatocarcinoma (HCC) in nude mouse model. Here we demonstrated that TTF1-NP inhibits tube formation of HUVECs and HepG2 cell migration and invasion, and inhibits tumor growth in nude mice implanted with HepG2 cells through the downregulation of STAT3 protein and activation, along with VEGF, KDR, bFGF, MMP2 and MMP9 levels. We further revealed that TTF1-NP decreased the DNA-binding capacity of STAT3. Together our results provide a mechanism by which TTF1-NP suppresses cancer cell migration, invasion and angiogenesis through the action of STAT3 and suggests TTF1-NP as a potential therapy for hepatocellular cancer treatment.

Keywords: angiogenesis; cell invasion; cell migration; Flavone derivative (TTF1); hepatoma; STAT3

1. Introduction

Hepatocellular carcinoma (HCC) is a primary liver cancer, the second leading cause of cancer mortality among the worldwide [1], and presents a major global health problem owing to its increasing incidence [2,3]. Clinical presentation is variable, however, most HCC patients will receive chemotherapy at some point during treatment as a means of prolonging life, most currently drugs show limited efficacy [4–6]. The toxicity and limited efficacy of current treatments for HCC patients has led to a significant research effort focused on developing drugs with fewer side effects and greater therapeutic efficacy.

Natural products have been used to treat cancer for more than 40 years, many medicines are natural products or their derivatives [7,8], and more than 60% of the clinically used anti-tumor compounds are natural products or are derived from these compounds. The medicinal plant Sorbaria sorbifolia (SS), commonly known as False Spiraea, has been traditionally used as an anticancer therapy in the area surrounding Changbai Mountain in China. We previously reported that 5,2′,4′-trihydroxy-6,7,5′-trimethoxyflavone (TTF1), an extract from SS, is the major anticancer bioactive constituent of SS, and TTF1 inhibited angiogenesis in chick embryo chorioallantoic membranes and induced HepG2 cells apoptosis [9,10].

However, TTF1 has been limited for use as a potential anticancer drug owing to its low absorbance and high biodegradability. To address these limitations, biodegradable and small molecule TTF1 nanoparticles (TTF1-NP) were prepared using an emulsion
evaporation-solidification method at low temperature [11]. TTF1-NP is highly soluble and inhibited the HepG2 cell growth in vitro and in a nude mouse model [12]. Although intensive research has led to considerable reduction of the limitations of TTF1, the anti-hepatoma effects of TTF1-NP and its underlying molecular mechanism has remained unknown. Herein, we evaluated the effects of TTF1-NP on hepatoma HepG2 cells and its anti-hepatoma mechanism. In addition, we also explored the potential molecular targets of TTF1-NP.

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2. Results

2.1. TTF1-NP Inhibited Human Hepatoma HepG2 Cells Growth In Vitro and In Vivo

We firstly examined the inhibitory effect of TTF1-NP on the growth of human hepatoma HepG2 cells in vitro. (Figure 1a). HepG2 cells were treated with TTF1-NP (25, 50, 100, 200 or 400 μM) or DMEM medium (Vehicle) for 48 h. We observed an increase of cell growth inhibition with increasing concentration of TTF1-NP, and the IC50 value of TTF1-NP against HepG2 was 98.26 μmol·L⁻¹ at 48 h. We further treated HepG2 cells with TTF1-NP (100 μM) for various time points (6, 12, 24, 36 and 48 h) and found a similar significant inhibition of cell growth rate (%) with increasing TTF1-NP concentration (Figure 1b). The results showed that TTF1-NP treatment significantly reduced the HepG2 cells growth in a time- and dose-dependent manner.

![Figure 1](https://example.com/figure1.png)

Figure 1. TTF1-NP inhibited human hepatoma HepG2 cells growth in vitro(a,b) and in vivo(c,d,e). (a) The cell growth inhibition ratio (%) for HepG2 cells were calculated after treatment with TTF1-NP in 25, 50, 100, 200, 400 μM or DMEM medium (Vehicle) for 48 h. Cell growth inhibition ratio (%) = (OD_{Vehicle} - OD_{treatment}) / OD_{Vehicle} × 100%; (b) The cell growth inhibition ratio (%) for HepG2 cells were calculated after treatment with TTF1-NP (100 μM) for 6, 12, 24, 36 and 48 or 0 h (Vehicle). Cell growth inhibition ratio (%) = (OD_{Vehicle} - OD_{treatment}) / OD_{Vehicle} × 100%; (c) Dynamic changes in gross tumor volume of tumors from HepG2 cells implanted into nude mice after treatment with TTF1-NP for 5, 10, and 20 μmol·kg⁻¹, gross tumor volume = 1/2 (length × width); (d) Volumes of tumors (cm³) were calculated after treatment with TTF1-NP for 5, 10, and 20 μmol·kg⁻¹; (e) Tumor growth inhibition ratio (%) were calculated after treatment with TTF1-NP for 5, 10, and 20 μmol·kg⁻¹, tumor growth inhibition ratio (%) = (TW_{Vehicle} - TW_{treatment}) / TW_{Vehicle} × 100%; (f) Chemical structure of TTF1-NP. Results are presented as mean ± SD from five or six independent tests with triplicate samples. *p<0.05, **p<0.01 for the designated treatment vs. Vehicle.
We subsequently examined the effects of TTF1-NP on growth of tumors from implanted HepG2 cells in nude mice. Tumors from TTF1-NP-treated mice (5, 10, and 20 μmol·kg⁻¹) were significantly smaller than that of the Vehicle group mice (Figure 1c,d). In addition, the tumor growth inhibition ratio of the TTF1-NP-treated mice was significantly increased compared with Vehicle group mice ($p<0.01$) (Figure 1e). These results show that TTF1-NP inhibited human hepatoma HepG2 cells growth in vitro and in vivo.

2.2. TTF1-NP Inhibited HUVEC Tube Formation and HepG2 Cell Migration and Invasion, and Downregulated the Expression Levels of Related Proteins

To systematically assess the anti-tumor activity of TTF1-NP, we first evaluated its effects on HUVEC tube formation and on migration and invasion of HepG2 cells. As shown in Figure 2a, HUVEC tube formation was markedly decreased in response to TTF1-NP in a dose-dependent manner. TTF1-NP also inhibited the migration and invasion abilities of HepG2 cells in a dose-dependent manner (Figure 2b–e).

![Figure 2. TTF1-NP inhibited HUVEC tube formation and HepG2 cell migration and invasion and downregulated the expression levels of related proteins. (a) Tube formation assay with HepG2 cells treated with indicated concentration of TTF1-NP (100× magnification); (b) Transwell assays with HepG2 cells treated with indicated concentration of TTF1-NP (200× magnification); (c) Scratch healing assay with HepG2 cells treated with indicated concentration of TTF1-NP (100× magnification); (d) Invasion of HepG2 cells treated with TTF1-NP in transwell assays (b), quantitative data of migrated cells represent the mean ± SD of three measurements, *$p<0.05$, **$p<0.01$ for the designated treatment vs. Vehicle; (e) Migration of HepG2 cells treated with TTF1-NP in scratch healing assays (c), quantitative data of migrated cells represent the mean ± SD of three measurements, *$p<0.05$, **$p<0.01$ for the designated treatment vs. Vehicle; (f) Western blot analysis of VEGF, KDR, bFGF, MMP2 and MMP9 expression in HepG2 cells treated with TTF1-NP; (g) Quantification of western blot analysis of VEGF, KDR, bFGF, MMP2 and MMP9 protein levels normalized by the levels of β-actin in (f). Data represent mean ± SD of three independent experiments. *$p<0.05$, **$p<0.01$ for the designated treatment vs. Vehicle.](image-url)
VEGF, KDR, bFGF, MMP2 and MMP9 are critical factors involved in tube formation and cell migration and invasion. To more closely examine the anti-angiogenic effects of TTF1-NP, we next analyzed VEGF, KDR, bFGF, MMP2 and MMP9 levels in HepG2 cells. As shown in Figure 2f, the proteins’ expression levels were all significantly lower in the TTF1-NP-treatment groups compared with the Vehicle group and significantly correlated with dosage. Together these results show that TTF1-NP inhibited HUVEC tube formation and HepG2 cell migration and invasion and downregulated the expression levels of related proteins.

### 2.3. TTF1-NP Downregulated STAT3 and p-STAT3 Expression in Different HCC Cell Lines

STAT3 plays a major role in tumor cell survival, proliferation, metastasis, and angiogenesis. Activation of STAT3 requires the phosphorylation of STAT3 at tyrosine 705 (Tyr705). Thus, we next investigated whether TTF1-NP impacts STAT3 activation in several human hepatoma cell lines (HepG2, Hep3B, PLC/PRF/5, and SMMC-7721). We observed partial downregulation of STAT3 and p-STAT3 in various HCC cell lines treated with TTF1-NP (100 μM) compared with the Vehicle groups (Figure 3a–c). Notably, the inhibitory effects of TTF1-NP on STAT3 and p-STAT3 expression were statistically significant in HepG2 cells. We thus used HepG2 cells for subsequent experiments.

![Figure 3](image)

**Figure 3.** TTF1-NP downregulated expression of STAT3 and p-STAT3 in different HCC cell lines. (a) Western blot assays the expression of STAT3 and p-STAT3 in different HCC cell lines; (b,c) Quantification of western blot analysis of (b) p-STAT3 and (c) STAT3 levels normalized by the levels of β-actin. Data represent mean ± SD of three independent experiments.*p< 0.05 for the designated treatment vs. Vehicle.

### 2.4. TTF1-NP Inhibited Expression of STAT3 and p-STAT3 in Human Hepatoma Cells In Vitro and In Vivo

Because TTF1-NP showed a significant inhibitory effect on HepG2 cell growth, we tested the effects of TTF1-NP on the expression and location of STAT3 and p-STAT3 in vitro and in vivo. We found that STAT3 and p-STAT3 protein expression was significantly reduced in response to TTF1-NP in HepG2 cells in a dose-dependent manner (Figure 4a–c). Similar results were observed in tumors from nude mice implanted with HepG2 cells (Figure 4d–f). The levels decrease as the number of positive cells decrease in TTF1-NP treatment group compared with the Vehicle group in both HepG2 cells and nude mice (p< 0.05) (Figure 4b,e). Immunohistochemical staining of untreated tumors showed that STAT3 protein localized in the cytosol while p-STAT3 protein was clearly localized in cell nucleus (Figure 4c,f). Together these results show that TTF1-NP treatment induced a decrease in the levels of STAT3 protein in both HepG2 cells and tumor from nude mice.
Figure 4. TTF1-NP inhibited expression of STAT3 and p-STAT3 in human hepatoma cells in vitro (a,b,c) and in vivo (d,e,f). (a,d) Western blot analysis of STAT3 and p-STAT3 after TTF1-NP treatment in (a) HepG2 cells and (d) nude mice implanted with HepG2 cells; (b,e) Quantification of western blot analysis of STAT3 and p-STAT3 protein levels normalized by the levels of β-actin in (b) HepG2 cells and (e) nude mice implanted with HepG2 cells, data represent mean ± SD of three independent experiments. *p < 0.05 for the designated treatment vs. Vehicle; (c,f) Immunocytochemistry of STAT3 and p-STAT3 in (c) HepG2 cells and (f) nude mice implanted with HepG2 cells after TTF1-NP treatment (200× magnification).

2.5. STAT3 Played a Role in TTF1-NP Inhibited Cell Angiogenesis, Migration and Invasion

To determine whether the decrease in STAT3 induced by TTF1-NP was responsible for the inhibition of angiogenesis and cells migration and invasion, we performed experiments silencing STAT3 using siRNA. We first tested several siRNA targeting STAT3 and confirmed that STAT3-siRNA3 (5′AUAAAGCCCAUGAUGUACCTT 3′) markedly reduced STAT3 levels and activation in HepG2 cells (Figure 5a,c). As shown in Figure 5b,d, treatment of HepG2 cells with TTF1-NP (100 μM) or STAT3 siRNA3 alone both caused a significant reduction of the expression in STAT3 and p-STAT3. However, treatment of TTF1-NP in STAT3-siRNA3 HepG2 cells did not cause further significant reduction as compared with cells treated with alone, suggesting that TTF1-NP has a potential target effect on inhibition the activation of STAT3.

We demonstrated that TTF1-NP caused cells functional inhibition in HepG2 through downregulations of STAT3 above, we next examined the effects of TTF1-NP in HepG2 cells transfected with STAT3 siRNA3. Blockade of STAT3 alone induced capillary structure formation and inhibition of cell invasion in HepG2 cells (Figure 5e–g). However, treatment with TTF1-NP in...
STAT3-siRNA3 HepG2 cells did not cause further inhibition compared with STAT3 siRNA3 transfected cells (p>0.05) (Figure 5h,i). We also examined the expression of VEGF, KDR, MMP2 and MMP9 and found downregulation of all examined proteins in HepG2 cells with blocked STAT3 expression compared with the Vehicle group, treatment with TTF1-NP in STAT3-siRNA3 HepG2 cells did not cause further significant inhibition compared with STAT3 silenced cells (p>0.05) (Figure 5j,k). These results suggested that TTF1-NP has a potential target effect on inhibition the activation of STAT3, inhibited the activation of STAT3 can suppress cells angiogenesis and migration and invasion.

Figure 5. STAT3 plays a role in TTF1-NP inhibition of cell angiogenesis, migration and invasion. (a) Western blot analysis suppression of STAT3 and p-STAT3 protein in HepG2 cells knocked down for STAT3 by siRNA; (b) Western blot analysis STAT3 and p-STAT3 expression in cells transfected...
with STAT3–siRNA or negative control siRNA (NC) and treated with TTF1-NP as indicated; (c,d) Quantification of western blot data from (a,b), data represent mean ± SD of three independent experiments. *p< 0.05 and **p< 0.01 for the designated treatment vs. Vehicle; (e) Tube formation assay with HepG2 and STAT3-siRNA HepG2 cells treated with TTF1-NP in 100μM (100× magnification); (f) transwell assays with HepG2 and STAT3-siRNA HepG2 cells treated with TTF1-NP in 100μM (200× magnification); (g) Scratch healing assay with HepG2 and STAT3-siRNA HepG2 cells treated with TTF1-NP in 100μM (100× magnification); (h) Quantitative data of migrated cells in (f), represent the mean ± SD of three measurements, *p< 0.05 and **p< 0.01 for the designated treatment vs. Vehicle; (i) Western blot analysis of VEGF, KDR, MMP2 and MMP9 expression in cells treated as indicated; (k) Quantification of western blot analysis normalized by the levels of β-actin. Data represent mean ± SD of three independent experiments. *p< 0.05 and **p< 0.01 for the designated treatment vs. Vehicle.

2.6. TTF1-NP Suppressed Angiogenesis and Cells Migration in STAT3 Over-Expressed HepG2 Cells

To conversely verify the potential target effect of TTF1-NP on inhibition the activation of STAT3, we Over-expressed STAT3 in HepG2 cells and found that TTF1-NP inhibited the activation of STAT3 in STAT3 Over-expressing HepG2 cells (Figure 6a,b). Over-expression of STAT3 in HepG2 cells caused a remarkable increase of capillary structure formation and cell invasion (Figure 6c,d). However, treatment of STAT3 Over-expression cells with TTF1-NP partially inhibited this effect. In addition, we examined the expression of VEGF, KDR, MMP2 and MMP9 in STAT3 Over-expressing HepG2 cells and found an upregulation of all examined proteins compared with the Vehicle group (Figure 6f,g). Treatment with TTF1-NP inhibited this induction of protein expression compared with the STAT3 Over-expression group. Taken together, STAT3 is an important target that TTF1-NP suppressed angiogenesis and cells invasion in STAT3 Over-expression HepG2 cells.

*Figure 6. TTF1-NP suppressed angiogenesis and cell migration in STAT3 Over-expressing HepG2 cells. (a) The STAT3 and p-STAT3 protein levels in HepG2 cells transfected with STAT3 plasmid and
treated with TTF1-NP as indicated; (b) Quantification of western blot analysis in (a), normalized by the levels of β-actin, *p < 0.05 for the designated treatment vs. Vehicle, †p < 0.05 for the STAT3-Overexpression+TTF1-NP group vs. STAT3-Overexpression group; (c) Tube formation assays with HepG2 and STAT3 Over-expression HepG2 cells treated with TTF1-NP in 100μM (100× magnification); (d) Transwell assays with HepG2 and STAT3 Over-expression HepG2 cells treated with TTF1-NP in 100μM (200× magnification); (e) Quantification of transwell assays in (d), *p < 0.05 for the designated treatment vs. Vehicle, †p < 0.05 for the STAT3-Overexpression+TTF1-NP group vs. STAT3-Overexpression group; (f) Western blot analysis of VEGF, KDR, MMP2 and MMP9 expression in HepG2 cells treated as indicated; (g) Quantification of western blot analysis of protein levels normalized by the levels of β-actin, *p < 0.05 for the designated treatment vs. Vehicle, †p < 0.05 for the STAT3-Over-expression+TTF1-NP group vs. STAT3-Over-expression group.

2.7. TTF1-NP Inhibited STAT3 DNA Binding Activity

To examine the effect of TTF1-NP on STAT3 activity, we assayed STAT3 DNA binding activity using EMSA. EMSA results showed that TTF1-NP inhibited STAT3 DNA-binding activity in a dose-dependent manner (Figure 7a,b).

![Figure 7](image)

**Figure 7.** Effect of TTF1-NP on STAT3 DNA binding activity in HepG2 cells. (a) EMSA assays with HepG2 cells combined with TTF1-NP in 50, 100, 200μM; (b) Quantification of EMSA results using Image Quant software (Amersham). Data represent the mean±SD of three measurements. *p < 0.05, **p < 0.01 vs. Vehicle group.

3. Discussion

TTF1 is the major anticancer bioactive constituent of SS, which is traditionally used as an anticancer therapy in the area surrounding Changbai Mountain in China. To make TTF1 more conducive to organism absorption, we prepared biodegradable small molecule TTF1-NP [11]. We previously demonstrated the anti-proliferation effect of TTF1-NP in human hepatoma cells [12]. In this study, we more closely examined the anti-proliferation action of TTF1-NP in human hepatoma cells.

To investigate the effect of TTF1-NP on STAT3 in HCC, we assessed STAT3 expression in several HCC cell lines treated with TTF1-NP. We found that STAT3 and p-STAT3Tyr705 protein expressions were decreased in different HCC cell lines. STAT3 is a transcription factor, known as an attractive target for cancer therapy. Several studies have demonstrated that directly targeting STAT3 represents a potential therapeutic approach to treating cancer [13–15]. STAT3 activation occurs upon phosphorylation of Tyr705, leading to dimerization and translocation from the cytoplasm to the nucleus [16]. Persistent activation of STAT3 is involved in pathways involving tumorigenesis, cell proliferation and angiogenesis [17–20].
For deeper study of TTF1-NP on STAT3, we chose HepG2 cells for subsequent research. We found that TTF1-NP showed significantly inhibition on HepG2 cells and tumor growth in nude mice, as well as a significant inhibitory effect on the expressions of STAT3 and p-STAT3, which correlated with the treatment dose of TTF1-NP in HepG2 cells and nude mice with implanted HepG2 cells. These findings suggest that TTF1-NP inhibited cell proliferation and downregulated STAT3 and p-STAT3 expression in HCC cells.

To demonstrate the antitumor effects of TTF1-NP, we performed tube formation assay, transwell assay and scratch healing assay. We found that tube formation was markedly decreased in response to TTF1-NP in a dose-dependent manner, confirming our previous results showing that TTF1 inhibited on angiogenesis in chorioallantoic membrane. We also found that TTF1-NP inhibited the invasion and migration of HepG2 cells in a dose-dependent manner. Furthermore, we found significantly lower VEGF, KDR, bFGF, MMP2 and MMP9 proteins expression levels in the TTF1-NP-treatment group than the Vehicle group. Recent studies show that STAT3 can directly activate VEGF, which results in the initiation of cell metastasis and angiogenesis [21–23].

Angiogenesis is one of the important ‘hallmarks’ of cancer and an important factor in the proliferation, metastasis, and drug sensitivity of human neoplasms [24]. Therapies that block angiogenesis may help inhibit tumor growth and metastasis. Compared with chemotherapy, antiangiogenic therapy may have many advantages [25,26].

Angiogenesis is tightly controlled by diverse subsets of ligands and receptors, and among the known angiogenic molecules, vascular endothelial growth factor (VEGF) is the best-characterized modulator of angiogenesis and metastatic growth in human carcinogenesis [27], and vascular endothelial growth factor receptor 2 (KDR) is the major effector of VEGF-stimulated cell survival and vascular permeability during angiogenesis [28,29]. As well as, MMPs family also plays an important role in tumour metastasis and angiogenesis.

MMP2 and MMP9 are critical members of this family, and MMP9 is directly associated with angiogenesis and metastatic processes [30,31]. Thus, we speculated that TTF1-NP inhibited the tube formation, the invasion and metastasis by inhibited the expression of VEGF, KDR, bFGF, MMP2 and MMP9 through inhibition of STAT3.

To determine the role of STAT3 in TTF1-NP inhibitory effects, we knocked down STAT3 with siRNA and Over-expressed STAT3 in HepG2 cells. We found that STAT3 can regulate cells angiogenesis, invasion and metastasis, and STAT3 is the main target of TTF1-NP inhibiting the HepG2 cell angiogenesis, invasion and metastasis. Actually, the activation of STAT3 is due to the phosphorylation of the tyrosine 705 site, which proximal C, STAT3 binds to STAT3-specific DNA binding elements and upregulates various STAT3 downstream target genes among them, VEGF was an important one [32], which create conditions for tumor invasion and metastasis. To more closely examine the inhibition effect by TTF1-NP, we examined the STAT3 DNA binding activity. We found TTF1-NP caused a statistically significant inhibition of STAT3 DNA binding activity and phosphorylation of STAT3.

According to the results of this study, we speculate that the antitumor mechanism of the TTF1-NP against HepG2 cells were like this: TTF1-NP can down-regulate the activities of STAT3 upon the phosphorylation of the tyrosine 705 site, as well as decrease STAT3 binds to STAT3-specific DNA binding elements. To extend that, the downstream target genes of STAT3 (VEGF, KDR, bFGF, MMP2 and MMP9) was inhibited obviously, so that the initiation of cell metastasis and angiogenesis were decreased. In view of the above, the tumor proliferation was significantly inhibited (Figure 8).
In summary, our results suggest that TTF1-NP has the potential to be a therapeutic agent for the treatment of human HCC, and STAT3 may be the potential therapeutic target of TTF1-NP. Future studies should address the limitations of TTF1-NP using the chemical modification and provide sufficient evidence for the efficacy of TTF1-NP against HCC in animal models to investigate the long-term efficacy of TTF1-NP and associated toxicity. Finally, we hope that the exploration of TTF1-NP can provide a theoretical basis for effective targeted therapy and drug screening for HCC.

4. Materials and Methods

4.1. Preparation of TTF1-NP

TTF1 was isolated from 10 kg of SS (collected from Yanji, Jilin Province, China) using water extraction and alcohol (WAE) precipitation as previously reported [9]. Biodegradable, small molecule (average particle diameter, 195.2 ± 35.2 nm; average entrapment efficiency, 64.57% ± 8.21%) TTF1-NP were prepared with a stearic acid solid lipid nanostructured carrier using an emulsion evaporation-solidification method at low temperature, as previously reported [11]. The TTF1-NP were highly soluble and TTF1 diffusion was characterized by an initial stage of burst release [10,11].

4.2. Cells and Culture Conditions

Human hepatoma cell lines (HepG2, Hep3B, PLC/PRF/5, and SMMC-7721) and HUVEC cells were purchased from Key GEN Co., Ltd. (Nanjing, China) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), 100U/mL penicillin (Gibco, USA), and 100 mg/L streptomycin (Gibco, USA) at 37 °C in a humidified incubator with a 5% CO₂ atmosphere.

4.3. Animals and Culture Conditions

BALB/c male nude mice (28 days, 15–17 g) were purchased from Shanghai Laboratory Animal Co., Ltd., (Shanghai, China). The tumor implantation experiments were authorized by the Institutional Animal Care and Use Committee of Yanbian University. The animals were housed in a laminar air-flow room maintained at a temperature of 22 ± 1 °C and a reactive humidity of 55% ± 1%.
4.4. Methods

4.4.1. MTT Assay

The effect of TTF1-NP treatment on cell growth was assessed in human hepatoma cell line (HepG2) using the MTT assay. The cells were treated with TTF1-NP (25, 50, 100, 200, or 400 μM) or DMEM (Vehicle) after 48 h. The cells were treated with TTF1-NP (100 μM) for 6, 12, 24, 36 and 48 h or 0 h (Vehicle). Briefly, the cells were exposed to MTT (5 g/L) for 4 h at 37 °C, 180 μL of DMSO was added, and the optical density (OD) was measured at 490 nm with a spectrophotometer (Thermo Fisher, Shanghai, China).

The cell growth inhibition ratio (%) was calculated as follows: Cell growth inhibition ratio (%) = \( \frac{OD_{Vehicle} - OD_{treatment}}{OD_{Vehicle}} \times 100\% \). where \( OD_{Vehicle} \) is the OD of the Vehicle group and \( OD_{treatment} \) is the OD of the treatment group. The results were also expressed as IC50 values.

4.4.2. Tube Formation Assay

50 μL matrigel (BD Bioscience, USA) was added to each well of 96-well plates (Corning, Inc., Acton, MA, USA) and the plates were incubated at 37 °C for 30 min for hardening. Then, 180 μL HUVEC suspensions \((2 \times 10^6 \text{ cells/mL})\) and different doses of the tested compounds were seeded on the Matrigel-coated plates for 12 h at 37 °C. Three different fields were chosen randomly in each well, and photographs were taken under the inverted microscope at 48h. Then photographed by a light microscope (Olympus, Tokyo, Japan) at 200× magnification.

4.4.3. Invasion Assay

The cell invasion ability was detected using a transwell membrane (8μm pore size, Costar, Acton, MA, USA) coated with Matrigel (BD Biosciences, San Diego, CA, USA). A total number of \(1 \times 10^5\) cells per well were cultured in the upper chamber, while 20% FBS was added to the lower compartment. Following 48 h incubation, non-invading cells were scraped by a cotton swab. The invaded cells were fixed with 100% methanol for 10 min, stained in 0.1% crystal violet for 30 min, and then photographed by a light microscope (Olympus, Japan) at 200× magnification, three different fields were chosen randomly in each well.

4.4.4. Scratch Healing Assay

Cells were seeded in a 6-well plate (Corning, Inc., USA) at a density of \(1 \times 10^6\) per well in 2 mL media and cultured overnight. At 60%–70% confluence, the cells were treated with different doses of the tested compounds for a further 48 h. Upon reaching 90% confluence, a scratch was created with a 100 μL-pipette tip and wells were washed three times with PBS to remove loose cells. Then cells were incubated in fresh medium and cell scratches were photographed by a light microscope (Olympus, Japan) at 100× magnification, three different fields were chosen randomly in each well.

4.4.5. siRNA Transfection

STAT3 siRNA and negative control (NC) were designed and synthesized by Shanghai Genechem Co., Ltd. (Shanghai, China). The HepG2 cells were transfected using Lipofectamine™2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. Briefly, the cells were seeded at \(2 \times 10^5\) cells per well and grown to 30%–50% confluence. Transfection complexes were prepared according to the manufacturer’s instructions and were added directly to the cells. After 4–6 h, transfection solution was replaced by DMEM medium with 10% FBS. The expression of STAT3 and p-STAT3 was confirmed by western blot after transfected 48 h.

4.4.6. Plasmid Construction and Transfection

pcDNA3.1 plasmid of STAT3 was synthesized by General Biosystems (An Hui, General Biosystems, Inc., Chuzhou, China). The HepG2 cells were transfected with pcDNA3.1-STAT3 using
Lipofectamine™2000 (Invitrogen, USA) according to the manufacturer’s protocol. Also, HepG2 cells were transfected with the empty vector (pcDNA3.1) to generate as a negative control group (NC). Cells were seeded in a 96-well plate (1×10^5 cells/mL). After cell attachment, adjust cell density to 50% when transfection. Transfection complexes was prepared according to manufacturer’s instructions and then added to the plate. After 4–6 h, transfection solution was replaced by DMEM medium with 10% FBS. The expression of STAT3 and p-STAT3 was confirmed by western blot after transfection 48 h.

4.4.7. Immunocytochemistry Staining Assay

Cells were seeded in a 6-well plate at a density of 1×10^6 cells/well in 2 ml media and cultured overnight. Then the cells were treated with TTF1-NP (50, 100 and 200 μM) or DMEM (Vehicle) after 48 h. Briefly, the cells were washed and blocked for 20 min in 3% hydrogen peroxide. The cells were incubated with primary antibodies overnight at 4°C and then with horseradish peroxidase-conjugated secondary antibodies for 30 min at 37°C. The cells were then developed with 3,3’-diaminobenzidine and counter-stained with hematoxylin. The cells were photographed by a light microscope (Olympus, Japan) at 200× magnification, three different fields were chosen randomly in each well. The following antibodies were used: anti-STAT3 (1:200 dilution; Abcam, Cambridge, UK, USA), anti-phospho-STAT3 (Tyr705) (1:500 dilution; Abcam, USA).

4.4.8. Western Blot Analysis

Total protein was extracted from the cells using RIPA lysis buffer (Solarbio, Beijing solarbio science & technology co.,ltd., Beijing, China), and the protein concentration was determined using a BCA Kit (Solarbio, China). Equal amount of the proteins was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membrane was blocked with 5% non-fat milk for 2 h and incubated with primary antibodies overnight at 4°C, followed by incubation with secondary antibodies for 2 h at 37°C (Table 1). Protein bands were detected using a BIO-RAD imaging system (BIO-RAD, Hercules, CA, USA). The intensity of each band was quantified by densitometry analysis using Image Pro Plus 4.5 software (4.5, Media Cybernetics, Inc., Bethesda, MD, USA).

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<tr>
<th>Antibody</th>
<th>Company</th>
<th>No.</th>
<th>Dilution</th>
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<tbody>
<tr>
<td>STAT3</td>
<td>Abcam</td>
<td>ab68153</td>
<td>1:1000</td>
</tr>
<tr>
<td>p-STAT3(Tyr705)</td>
<td>Abcam</td>
<td>ab76315</td>
<td>1:5000</td>
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<td>ab76003</td>
<td>1:1000</td>
</tr>
<tr>
<td>bFGF</td>
<td>Abcam</td>
<td>ab92337</td>
<td>1:1000</td>
</tr>
<tr>
<td>β-actin</td>
<td>Cell Signaling Tech.</td>
<td>12620s</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

4.4.9. Electrophoretic Mobility Shift Assay (EMSA)

Nuclear protein and cytoplasm protein was extracted from the cells (Key Gen Bio Tech, Nanjing, China), and the protein concentration was determined using a BCA Kit (Solarbio, China). The proteins were added to the reaction mixture prior to the DNA. All reaction mixtures were incubated on ice for 40 min. Then the reaction mixtures were resolved on a 6% pre-chilled non-denaturing polyacrylamide gel in Tris-borate-EDTA (TBE) buffer (pH 8.8) with or without the addition of 10 mM L-arginine at 100 V for 40 min. After electrophoresis at 4°C, the gel was visualized using an EMSA staining kit (Key Gen Bio Tech, China) and G: BOX chemiXR5 imaging.
system (Syngene, Cambridge, UK) The intensity was quantified by densitometry analysis using Gel-Pro 32 software (Media Cybernetics, Inc., Bethesda, MD, USA).

4.4.10. Tumor Implantation

HepG2 cells (1 × 10^6 cells/mL) were injected subcutaneously into the left posterior flank of male BALB/c nude mice (n = 6). The greatest longitudinal diameter (length) and greatest transverse diameter (width) were measured by caliper for each tumor to estimate gross tumor volume (GTV) as follows: GTV = 1/2 (length × width)^3. When the GTV reached 50–100 mm^3, the mice were randomly assigned to groups that received TTF1-NP (5, 10, or 20 μmol/kg), or normal saline (Vehicle) every other day for 20 days (10 intravenous administrations). GTV was measured 8, 10, 12, 14, 16, 18, and 20 days after the initiation of treatment. The animals were sacrificed 20 days after the initiation of treatment to measure tumor weight and volume, from which the tumor growth inhibition ratio were calculated. Tumor growth inhibition ratio (%) = (TWVehicle−TWtreatment)/TWVehicle × 100%, where TWVehicle and TWtreatment were the tumor weights of the Vehicle and tumor groups, respectively.

4.5. Statistical Analysis

The data were presented as means ± standard deviations (SD) three to six samples were analyzed in each experiment. Comparisons were subjected to t-test and ONE WAY analysis of variance (ANOVA). pvalue < 0.05 was considered statistically significant. Statistical Package for the Social Sciences (SPSS) 18.0 (SPSS Inc., Chicago, IL, USA) and Graph Pad Prism 5.0 software (Graph Pad Software, San Diego, California, USA) were used to analyze the results.

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Author Contributions: XueWu Zhang designed the experiments. Bin Xiao and DongJing Lin performed the experiments, analyzed the raw data, and wrote the manuscript. Xuan Zhang and MeiLan Zhang assisted with the experiments.

Conflicts of Interest: The authors have no conflicts of interest to declare.

Abbreviations

HCC    hepatocellular carcinoma
SS    Sorbaria sorbifolia
STAT3  Signal transducer and activator of transcription 3
TTF1-NP 5,2′,4′-tri hydroxy-6,7,5′-trimethoxyflavone nanoparticles
VEGF  vascular endothelial growth factor
KDR  vascular endothelial growth factor receptor 2
bFGF  basic fibroblast growth factor
MMP2  matrix metalloprotein 2
MMP9  matrix metalloprotein 9
siRNA  small interfering RNA
EMSA  electrophoretic mobility shift assay

References


Sample Availability: Samples of the compounds are available from the authors.