Iron deprivation suppresses hepatocellular carcinoma growth in experimental studies

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Keywords: Hepatocellular Carcinoma, Iron Deprivation, Thiosemicarbazone-24, HFE knockout mouse, xenograft mouse model

Running title: TSC24 suppressed HCC through iron deprivation

Translational Relevance

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related death, more than 80% of liver cancer cases occur in developing countries, such as China and Africa. There is an urgent need for novel anti-cancer drugs to improve the clinical
outcome in patients with HCC. Iron overload is a significant risk factor in the development of HCC. The present investigation was designed to evaluate a novel iron chelator, TSC24, and to determine its iron chelation efficiency and its anticancer effects on HCC. In the present study, we demonstrated that TSC24 chelated iron efficiently and showed potent anticancer effect on human HCC both in vitro and in vivo through iron deprivation. These results provide a rationale for the future development and applications of TSC24 or iron deprivation to treat patients with HCC.
Abstract

Purpose: Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related death and iron overload is a significant risk factor in the development of HCC. In this study we investigated the potential application of depriving iron by a novel iron chelator, thiosemicarbazone-24 (TSC24), in HCC treatment.

Experimental Design: Two HCC cell lines and HFE knockout (HFE-/-) mice were used to determine iron chelation efficiency of TSC24. The anticancer effects of TSC24 on HCC were analyzed in vitro and in athymic xenograft mouse models.

Results: Treatment with TSC24 significantly decreased the cellular iron concentration in hepatoma cells and the serum iron concentration in HFE-/- mice, by blocking iron uptake and interfering with normal regulation of iron levels. Moreover, the viability of HCC cell lines was reduced by TSC24. Confirming the mechanism of the agent, this decrease in viability could be partially rescued by addition of exogenous iron. TSC24 also suppressed tumor growth in athymic mice bearing human HCC xenografts in a concentration dependent manner without apparent toxicity in parallel with a decrease in the serum iron level. Further studies revealed that TSC24 efficiently triggered cell cycle arrest and apoptosis in Hep3B and HepG2 cell lines.

Conclusions: TSC24 is a potent iron chelator that suppresses human HCC tumor growth by disrupting iron homeostasis, reducing available iron and triggering cell cycle arrest and apoptosis, without apparent host toxicity at effective doses. Thus, TSC24 shows great potential for the treatment of HCC.
Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, ranking as the fifth most common cancer and the third most common cause of cancer-related death in the world (1). For the majority of patients who are not suitable for hepatic resection or transplantation, a few chemotherapeutic agents have been used to treat this disease, such as cisplatin, oxaliplatin, gemcitabine, and 5-fluorouracil. However, only a minority of patients respond to these drugs (2). Specific agents targeting molecules that are abnormally expressed in HCC may be more effective. For example, Sorafenib, a small molecular inhibitor of several tyrosine protein kinases, has recently been shown to provide a survival benefit in patients with early HCC (3-4). However, there is still an urgent need for novel anti-cancer drugs to improve the clinical outcome in patients with advanced HCC (5).

Iron is essential for both normal and cancer cells. It is required by many proteins involved in cell growth and proliferation. However, excess iron has been shown to increase cancer risk. Several studies in animal models clearly showed the carcinogenicity of excess iron (6-8). The role of iron in carcinogenesis has also been suggested from epidemiological studies which found that iron reduction by phlebotomy could prevent carcinogenesis in a supposedly normal population (6, 9-10).

In the body, iron is mainly stored in hepatocytes, making the liver the most sensitive organ affected by iron overloading (11-12). Indeed, iron overload in the liver represents a significant risk factor for development of HCC (11, 13-14).
animal model studies have provided evidence that excessive iron accumulation is a cause of HCC (12, 15-18). Patients with hereditary haemochromatosis (HH), most commonly caused by HFE mutation, show symptoms of profound iron overload and are at an increased risk of developing HCC (8, 19). Thus, it can be speculated that depriving cancer cells of essential iron could be an approach for cancer treatment. Indeed, iron chelators have been found to have antitumor activities (20).

To date, the most widely used chelator in clinical settings for the treatment of iron overload is desferrioxamine (DFO), and its anti-proliferative activity against neuroblastoma and leukemia has been examined in clinical trials (21). However, its application has been limited by several disadvantages including high cost, low oral bioavailability and the short serum half-life (21). Recently, new iron chelators with greater antitumor activities have emerged, such as Triapine (3-aminopyridine-2-carboxaldehyde thiosemicarbazone), DpT (di-2-pyridylketone thiosemicarbazone) and PKIH (di-2-pyridylketone isonicotinoyl hydrazone) analogs (20, 22-23). However, the therapeutic effect of iron chelators on human HCC has yet to be evaluated in either preclinical or clinical studies (http://clinicaltrials.gov/).

We previously designed and synthesized a series of \( \alpha \)-heterocyclic carboxaldehyde thiosemicarbazones, and TSC24 (Fig. 1) was shown to have the most potent anti-cancer activities among these compounds. Moreover, TSC24 is not only an iron chelator but also an inhibitor of topoisomerase II\( \alpha \) (24). In the current study, we investigated the iron chelation activity of TSC24 and its \textit{in vitro} and \textit{in vivo} effects on human HCC.
Materials and Methods

Cell culture and reagents.
HEK293 cells and human HCC cell lines, Hep3B and HepG2, were cultured in DMEM supplemented with 10% fetal bovine serum, and human non-neoplastic liver cell line 7702 was cultured in RPMI 1640 supplemented with 10% fetal bovine serum in a humidified incubator at 37°C and 5% CO₂. Cell viability was determined via MTT assay as described (25). TSC24 was synthesized as described previously (24). DFO, ferric ammonium citrate (FAC) and etoposide were purchased from Sigma-Aldrich, Inc. (St. Louis, MO).

Western blotting.
Western blot analysis was performed as described previously (25). Briefly, total cellular proteins were harvested using RIPA buffer, separated by SDS-PAGE, transferred to PVDF membranes, and probed with specific antibodies. Anti-human HIF1α, cleaved PARP were obtained from BD Biosciences (San Jose, CA), anti-human Caspase-3(8G10) was purchased from Cell Signaling Technology, Inc. (Danvers, MA) and anti-human β-actin(AC-74) was obtained from Sigma-Aldrich, Inc. All other antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Flow cytometric analysis.
HCC cells were treated with different concentrations of TSC24 for 24hr and analyzed for cell cycle and apoptosis using FACSCalibur (BD Biosciences) flow cytometry as described previously (25). To determine the effects of TSC24 on transferrin endocytosis,
cells were cultured in serum-free medium for 30 min and exposed to 50 μg/ml Alexa Fluor 633-conjugated transferrin (Invitrogen, Carlsbad, CA) for 2 hr. Then the cells were chilled on ice and washed with ice-cold PBS and harsh acid (0.2M acetic acid in 0.2M NaCl) to remove external transferrin. After cells were harvested, the mean fluorescence intensity was measured by flow cytometry. Data are normalized to the mean fluorescence in control cells.

Animals.

All animal studies were approved by the Institutional Animal Care and Use Committee of the Institute for Nutritional Sciences, Chinese Academy of Sciences (INS, CAS). BALB/c athymic mice (nu/nu) were purchased from Shanghai Slac Laboratory Animal Co. Ltd (Shanghai, China). HFE-/- mice were kind gifts from Prof. Fudi Wang, INS, CAS (with a C57/Bl/6-129/Ola genetic background, originally donated by Nancy C. Andrews, Departments of Medicine and Pediatrics, Harvard Medical School, Boston, MA). All animals were fed with commercial diet and water ad libitum.

Athymic xenograft mouse model and treatment.

Female BALB/c athymic mice (nu/nu) aged 3-4 weeks were used. The human tumor xenograft mouse models were established as described previously (25). 5×10^6 Hep3B or HepG2 cells were suspended in Matrigel (BD Biosciences) and injected s.c. into the left inguinal area. Mice bearing evident tumors were randomly divided into control and three treatment groups (five mice per group). TSC24 was dissolved in 57.1% PEG-400 in 14.3% ethanol plus 28.6% saline and was injected i.p. at a dose of 1, 5 or 10 mg/kg/d over
5 consecutive days per week for up to 3 weeks. Animals were sacrificed 24hr after the last dosing.

**HFE-/− mouse treatment and tissue iron measurement.**

HFE-/− mice at 6-8 weeks old were injected i.p. with vehicle or TSC24 at 20mg/kg/d for one week, and blood samples and liver, spleen specimens were collected for analysis. Serum iron was determined by colorimetric assay using the Iron/UIBC detection kit (Thermo Electron Corporation, Waltham, MA). Liver and spleen samples were accurately weighed, and digested in acid solution (3M hydrochloric acid and 0.61M trichloroacetic acid) for 48hr at 65°C, and mixed with chromagen stock: saturated sodium acetate: pure water (1:5:5, v/v/v; chromagen stock: 1.86mM bathophenanthroline sulfonate and 143mM thioglycollic acid). The absorbance was then recorded at 535nm using a SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA) and iron concentrations were calculated from a standard curve.

**Statistical Analysis.**

All data were expressed as the means ± SD unless otherwise indicated. The statistical significance of differences was examined using Student's t-test. Differences between groups were considered significant when $P < 0.05$. 
Results

**TSC24 chelated iron and disrupted iron uptake in HCC cell lines.**

The chemical structure of TSC24 is shown in Fig. 1. Infrared spectroscopic evidence showed that TSC24 can form a complex with iron (Supplementary Fig. S1), indicating that TSC24 is a novel iron chelator. To examine the effect of TSC24 on iron homeostasis in human HCC, Hep3B cells were incubated with TSC24 and the cellular iron concentration was determined. After treatment, the cellular iron level was significantly reduced compared to the control group (Fig. 2A). Considering that iron is internalized through binding to transferrin, we evaluated the effect of TSC24 on transferrin uptake. TSC24 decreased transferrin endocytosis in a concentration-dependent manner and this effect could be partially blocked by addition of an iron salt, ferric ammonium citrate (FAC) (Fig. 2B). Furthermore, TSC24 treatment diminished transferrin on cell membrane almost completely (Fig. 2C). These findings suggest that TSC24 may disrupt iron uptake and could, at least partially, explain why the cellular iron concentration was decreased by TSC24. In addition, TSC24 treatment re-distributed iron in Hep3B cells so that it was more concentrated compared with the control group (Supplementary Fig. S2).

**TSC24 affected signaling pathways of iron regulation in HCC cell lines.**

We examined the expression of several iron-responsive proteins in HCC cells. Incubation of Hep3B and HepG2 cells with TSC24 induced the expression of transferrin receptor-1 (TfR1) and hypoxia inducible factor-1α (HIF1α) in a concentration-dependent manner (Fig. 3A), which was a typical cell response to iron deficiency (26-27). The induced expression of HIF1α by TSC24 can be reversed by addition of FAC (Supplementary Fig.
Interestingly, expression of N-myc downstream regulatory gene-1 (Ndrg1) was downregulated by TSC24 (Fig. 3A), which was contrary to a previous observation with other iron chelators in some other types of cancer cells (28). Though Ndrg1 suppress metastasis in some cancers, Ndrg1 was oncogenic in HCC and knockdown Ndrg1 could inhibit tumor growth (29). This suggested that TSC24 may act in a tissue specific way. We also examined the expression of several genes involved in iron metabolism and showed that the mRNA levels of DMT1, Steap3, iron regulatory hormone Hepcidin and its upstream regulator BMP6 were all altered (Fig. 3B). The increases in BMP6 and decrease in Steap3 expression were observed both in TSC24- and in DFO- treated cells, and were reversible after supplementation of exogenous iron. In addition, TSC24 and DFO both markedly decreased the expression of Hepcidin but this could not be rescued by the addition of iron. TSC24, but not DFO, reduced DMT1 expression, and this could not be reversed either.

**TSC24 interfered with the iron distribution in HFE-/- mouse tissue.**

We chose HFE-/- mice, a typical animal model of iron overload, to further investigate the effect of TSC24 on iron disposition in vivo. After treatment with TSC24 for one week, the iron distribution in HFE-/- mouse tissue changed in a manner irrespective of sex (data not shown). There was a decrease in serum iron concentration (Fig. 4A). Consistent with other chelators, TSC24 increased splenic iron concentration (Fig. 4B) while did not change iron concentration in the liver (Fig. 4C). These results indicate that TSC24 also plays a role in the in vivo iron distribution and uptake, and that it can alter tissue distribution of iron in HFE-/- mice.
TSC24 exerted potent anticancer activities against HCC cell lines and xenograft HCC tumors through iron deprivation.

Based on the aforementioned iron chelation activity of TSC24, we speculated that this compound might have anti-cancer effects, and therefore implications for HCC therapy. We first examined the cytotoxicity of TSC24 in human HCC cell lines Hep3B (p53 null) and HepG2 (p53 wt), and non-neoplastic human liver cell line 7702. Treatment with TSC24 resulted in a significant decrease in the cell viability of both Hep3B and HepG2 but not 7702 cells (Fig. 5A). The IC_{50} values were 76.5nM (Hep3B), 74.1nM (HepG2) and >1000nM (7702), indicating that TSC24 selectively inhibits human HCC cells. More interestingly, the cytotoxicity of TSC24 could be reduced effectively by addition of exogenous iron (Fig. 5A), suggesting that, irrespective of p53 status, TSC24 exerts a profound anticancer activity at least partially through chelation of iron. We further evaluated the anti-cancer activity of TSC24 in vivo in athymic mice bearing Hep3B and HepG2 xenografts. TSC24 significantly inhibited tumor growth in a dose-dependent manner (Fig. 5B). The inhibition rates at Day 21 in the high dose group (10mg/kg) were 55.2% (Hep3B) and 57.7% (HepG2) (P < 0.01). TSC24 had little impact on mouse body weight (Fig. 5C) and no other evident physical abnormalities were observed. Further analysis showed that TSC24-treated athymic mice had a lower iron concentration in serum (Fig. 5D) and xenograft tumors (data not shown) compared with control mice.

To investigate whether topoisomerase IIα contributed to the effect of TSC24 on HCC, we used etoposide to inhibit and siRNA to knockdown topoisomerase IIα. And we found that, in both Hep3B and HepG2 cells, neither etoposide nor siRNA could reverse
the cytotoxicity of TSC24 (Supplementary Fig. S4A and B). Furthermore, we analyzed
the DNA damage signaling which would be activated in case of topoisomerase IIα
inhibition. And we found that etoposide, as the positive control, reduced the total levels
of Chk1 and Chk2, and increased γH2AX levels markedly, while TSC24 showed little
impact on them (Supplementary Fig. S4C). Also, we examined the mRNA expression of
topoisomerase IIα in both cell lines and tumor xenografts after TSC24 treatment, and
didn’t observe significant change (Supplementary Fig. S4D and E). These results
suggested that TSC24 exerted the anticancer activity to human HCC independent of
topoisomerase IIα inhibition.

**TSC24 arrested cell cycle in G2/M and induced apoptosis in HCC cell lines.**

We have demonstrated that TSC24 has efficient anti-cancer effects on HCC that are at
least partially mediated by its chelation of iron. To explore its mechanism(s) in greater
detail, we determined the effect of TSC24 on apoptosis and cell cycle progression in
HCC cell lines. As expected, we found that TSC24 induced apoptosis of Hep3B and
HepG2 cells in a concentration-dependent manner (Fig. 6A), and modulated signaling
pathways involved in apoptosis. Pro-apoptotic molecules p53, bax, cleaved PARP and
caspase-3 were up-regulated, while anti-apoptotic proteins MDM2 and bcl2 were
decreased by TSC24 (Fig. 6C). Moreover, TSC24 arrested cell cycle in G2/M phase in
both Hep3B and HepG2 cells (Fig. 6B). Expression of p27 and p21 were found to be
induced and Cdc25, cyclin A, cyclin B and E2F1 were down-regulated (Fig. 6C).
Histological analysis also indicated that TSC24 treatment induced apoptosis and inhibited
proliferation in tumor tissues (Supplementary Fig. S5).
Discussion

In the present study, we have demonstrated that TSC24, a novel iron chelator, showed efficient iron chelating activity and potent anticancer effect on human HCC both in vitro and in vivo. We speculate that TSC24 causes iron deprivation by chelating iron, disrupting iron uptake and impairing iron regulation. Iron deprivation in turn induces cell cycle arrest by regulating G2/M checkpoint proteins, and induces apoptosis through caspase-dependent pathway to suppress tumor. Our studies not only provide a rationale for the future development of this compound to treat HCC, but also provide mechanistic information on the use of iron deprivation for HCC therapy.

It has been well-documented that iron homeostasis and iron regulatory pathways are abnormal in human HCC (30-32). Thus, it is likely that iron chelators targeting excess iron may suppress HCC and bring clinical benefits to HCC patients. However, only a few iron chelators have been evaluated in clinical trials for cancer therapy (33-34), mainly due to the low efficiency of iron chelation in vivo as well as the toxicity. More effective chelators have now been developed. Among these, Triapine has entered phase I and II clinical trials for many types of cancer, but has not been tested for HCC (http://clinicaltrials.gov/). Another novel chelator, Dp44mT showed more potent anticancer activities than Triapine in preclinical studies (21, 35-36). However, studies of the therapeutic application of iron chelator for HCC have not been reported in either preclinical or clinical studies. In the present study, we focused on the iron chelating efficiency and anti-cancer potency against HCC of our novel iron chelator, TSC24, which is a thiosemicarbazone compound as Triapine and Dp44mT.
HFE-/- mice provides an animal model of Hereditary Hemochromatosis (HH), a most common iron overload disorder in humans (37-38). We found that TSC24 caused iron concentration to increase in spleen and decrease in serum. However, the hepatic iron concentration did not change. This may be due to the short period of treatment (1 week). In other settings, hepatic iron content decrease could be observed only in longer term treatments (≥7 weeks) but not in short term treatments (≤2 weeks) (20, 39). Nevertheless, the iron concentration in HCC cells was reduced after TSC24 treatment. In addition, the mechanisms by which TSC24 deprived HCC cells of iron are still unclear. Based on our observations, we speculate that complexing of iron with TSC24 may impair its binding to transferrin and subsequent internalization. Moreover, TSC24 may also alter the expression of several genes involved in iron metabolism and regulation. The role of iron deprivation in the anti-cancer effects of TSC24 was confirmed by iron rescue studies.

In fact, iron deprivation has been found to affect many proteins involved in cell cycle progression and apoptosis, eventually leading to cell cycle arrest and increased apoptosis (40). Moreover, we found that TSC24 could trigger cell cycle arrest and induce apoptosis in HCC cells. In another study, we found that TSC24 could induce cell cycle arrest at G1/S checkpoint in colon cancer cell lines (24), which is consistent with previous reports of most iron chelators (40-42). Surprisingly, we found that TSC24 treatment induced G2/M arrest in both Hep3B and HepG2 cells, indicating that the mechanism of action of TSC24 on cell cycle in HCC may be different from that in other types of malignancy. Immunoblot results further supported this observation. Furthermore, TSC24 treatment
potently induced apoptosis in HCC cells. All of the above observations were found to occur independent on p53 status of the cells. We also observed potent cytotoxicity of TSC24 in Huh7 cells (data not shown), another HCC cell line carrying a HFE mutation similar to that associated with human HH (43). Our findings suggest that TSC24 exerts its anti-cancer effects in a HFE-independent manner. While the cytotoxicity of TSC24 is independent of a number of factors including cell type, p53 and HFE, it is specifically determined by iron. The addition of exogenous iron salt reversed the cytotoxicity of low concentration of TSC24 almost completely.

The reversal effect of exogenous iron on high concentration of TSC24 (>100nM) was not significant, indicating that TSC24 may suppress HCC through other ways in addition to iron-chelating. One alternative mechanism is the possible chelation of other divalent metal ions by TSC24 (such as Magnesium, Zinc et al). The other alternative mechanism is the inhibition on topoisomerase IIα. We have shown that TSC24 inhibited the catalytic activity of topoisomerase IIα at high concentration (≥1 μM) previous (24).

In conclusion, we have herein demonstrated that a novel iron chelator, TSC24, efficiently causes iron deprivation in HCC cell lines and in HFE-/- mouse model. Moreover, TSC24 was highly effective for suppressing HCC both in vitro and in xenograft mouse models. Our findings provide preclinical evidence to show that iron deprivation by iron chelators may have important implications in human HCC therapy, and indicate that TSC24 could be a promising compound for the treatment of human HCC. These possibilities warrant further investigation.
References


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Figure legends

Fig. 1. Chemical structure of TSC24.

Fig. 2. Effects of TSC24 on cellular iron concentration, transferrin uptake and localization. (A) Hep3B cells treated with DMSO or 2µM TSC24 for 2hr were harvested and cellular iron concentrations were measured by Atomic Absorption Spectrometry. Histograms showed the results of two experiments. *p < 0.05 compared with control cells. (B) HepG2 cells were treated and then incubated with Alexa fluor 633-conjugated transferrin. Harvested cells were subjected to flow cytometric analysis for internalized transferrin. The results were representative of three different experiments. *p < 0.05, **p < 0.01. (C) HEK293 cells were treated with DMSO or TSC24 for 2hr and incubated with Alexa fluor 633-conjugated transferrin. Cells were fixed and analyzed by confocal imaging.

Fig. 3. TSC24 impaired cell signaling regulating iron homeostasis in HCC cell lines. (A) Hep3B and HepG2 cells were treated with different concentrations of TSC24 for 24hr, and western blots were performed for protein detection. The amount of protein was quantified by densitometry, normalized to the amount of β-actin and shown below. (B) HepG2 cells with different treatments for 24hr were harvested, and mRNA levels were determined by quantitative RT-PCR. The results represented three different experiments. *p < 0.05, **p < 0.01.

Fig. 4. TSC24 modulated tissue iron distribution in HFE-/- mice. HFE-/- mice were injected i.p. once per day with vehicle or TSC24 (20mg/kg) for one week and the iron
concentrations in (A) serum, (B) spleen and (C) liver were determined. The means ± SEM of at least four animals in each treatment group are shown. *\( p < 0.05 \), **\( p < 0.01 \) compared with vehicle-treated mice.

**Fig. 5. In vitro and in vivo anticancer activity of TSC24 against HCC.** (A) 7702, Hep3B and HepG2 cells were treated with different concentrations of TSC24 in the presence or absence of 20\( \mu \)M FAC for 48hr, and cell viability was evaluated by MTT assay. The results represented at least three independent experiments. **\( p < 0.01 \) compared with cells treated with TSC24 only. (B) Inhibition of tumor growth in athymic mice bearing Hep3B or HepG2 xenografts. Tumor size was measured every three days. *\( p < 0.05 \), **\( p < 0.01 \) compared with vehicle-treated mice. (C) The body weight of mice treated with the different doses of TSC24 or vehicle was monitored every three days. (D) The serum iron concentrations in athymic mice bearing Hep3B xenografts treated with TSC24 (10mg/kg) or vehicle were examined. *\( p < 0.05 \) compared with vehicle-treated mice. All data from in athymic mice are shown as the means ± SEM.

**Fig. 6. Effects of TSC24 on apoptosis and cell cycle progression in HCC cell lines.** Hep3B and HepG2 cells were treated with different concentrations of TSC24 for 24hr and harvested for apoptosis analysis (A) and cell cycle distribution (B) by flow cytometry. The results of three independent experiments were shown. *\( p < 0.05 \), **\( p < 0.01 \) compared with control cells. (C) Total proteins were harvested and western blots were performed. The amount of protein was quantified by densitometry, normalized to the amount of GAPDH and shown below.
Figure 1

[Chemical structure image]

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Figure 2

(A) Relative iron concentration (% of ctrl)

(B) Transferrin uptake (% of ctrl)

(C) Picture showing transferrin localization in Ctrl and TSC conditions.

Ctrl vs TSC: *p < 0.05, **p < 0.01.
Figure 3

A

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**TSC**

- **FAC**
- **DFO**

*Significant difference compared to control.*

**Significant difference compared to control.**

*Significant difference compared to control.*
Figure 4

A

Serum iron (µg/DL)  
6 weeks 8 weeks  
Ctrl TSC  
**

B

Splenic iron (µg/g)  
6 weeks 8 weeks  
Ctrl TSC  
*

C

Hepatic iron (µg/g)  
6 weeks 8 weeks  
Ctrl TSC
Figure 5

A

7702

Hep3B

HepG2

Cell viability (%) vs. Concentration (nM)

- TSC only
- TSC+FAC

B

Hep3B

HepG2

Tumor mass (mg) vs. Day

- Saline
- TSC(1mg/kg)
- TSC(5mg/kg)
- TSC(10mg/kg)

C

Hep3B

HepG2

Body weight (g) vs. Day

- Saline
- TSC(1mg/kg)
- TSC(5mg/kg)
- TSC(10mg/kg)

D

Hep3B

Serum iron (µg/dL)

- Ctrl
- TSC

* P < 0.05
** P < 0.01
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