Am J Cancer Res 2021;11(6):2975-2989
www.ajcr.us /ISSN:2156-6976/ajcr0131236

Original Article

Pterostilbene inhibits hepatocellular carcinoma proliferation and HBV replication by targeting ribonucleotide reductase M2 protein

Rui Wang[1,2*], Zhijian Xu[3*], Jiaping Tian[1,2], Qian Liu[1], Jingwen Dong[1], Lijuan Guo[1], Boning Hai[1], Xia Liu[1], Hangping Yao[4], Zhi Chen[4], Junjie Xu[5], Lijun Zhu[6], Haiyi Chen[7], Tingjun Hou[7], Weiliang Zhu[3], Jimin Shao[1,2]

[1]Department of Pathology & Pathophysiology, and Cancer Institute of The Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China; [2]Zhejiang University Cancer Center, Key Laboratory of Disease Proteomics of Zhejiang Province, Key Laboratory of Cancer Prevention and Intervention of China National Ministry of Education, Zhejiang University School of Medicine, Hangzhou, China; [3]Drug Discovery and Design Center, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, China; [4]State Key Laboratory of Infectious Disease Diagnosis and Treatment, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China; [5]Department of General Surgery, Sir Run-Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou, China; [6]Key Laboratory of Pancreatic Disease of Zhejiang Province, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China; [7]Hangzhou Institute of Innovative Medicine, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou, China. [*]Co-first authors.

Received February 2, 2021; Accepted May 10, 2021; Epub June 15, 2021; Published June 30, 2021

Abstract: Hepatocellular carcinoma (HCC), one of the most deadly diseases all around the world. HBV infection is a causative factor of HCC and closely associated with HCC development. Ribonucleotide reductase (RR) is a key enzyme for cellular DNA synthesis and RR small subunit M2 (RRM2) is highly upregulated in HCC with poor survival rates. We have previously shown that HBV can activate the expression of RRM2 and the activity of RR enzyme for the viral DNA replication in host liver cells. Thus, RRM2 may be an important therapeutic target for HCC and HBV-related HCC. Pterostilbene, a natural plant component, potently inhibited *in vitro* RR enzyme activity with the IC_{50} of about 0.62 μM through interacting with RRM2 protein, which was much higher than current RRM2 inhibitory drugs. Pterostilbine inhibited cell proliferation with an MTT IC_{50} of about 20-40 μM in various HCC cell lines, causing DNA synthesis inhibition, cell cycle arrest at S phase, and accordingly apoptosis. On the other hand, the compound significantly inhibited HBV DNA replication in HBV genome integrated and newly transfected HCC cells, and the EC_{50} for inhibiting HBV replication was significantly lower than the IC_{50} for inhibiting HCC proliferation. Notably, pterostilbene possessed a similar inhibitory activity in sorafenib and lamivudine resistant HCC cells. Moreover, the inhibitory effects of pterostilbine against HCC proliferation and HBV replication were significantly reversed by addition of dNTP precursors, suggesting that RR was the intracellular target of the compound. Finally, pterostilbine effectively inhibited HCC xenograft growth with a relatively low toxicity in nude mouse experiments. This study demonstrates that pterostilbene is a novel potent RR inhibitor by targeting RRM2. It can simultaneously inhibit HCC proliferation and HBV replication with a potential new use for treatment of HCC and HBV-related HCC.

Keywords: Hepatocellular carcinoma (HCC), hepatitis B virus related hepatocellular carcinoma (HBV-related HCC), pterostilbene, ribonucleotide reductase small subunit M2 (RRM2), DNA synthesis inhibition

Introduction

Hepatocellular carcinoma (HCC) is one of the most deadly diseases all around the world [1]. Hepatitis B virus (HBV) infection is the leading cause of incident cases of liver cancer and deaths, especially in East Asia and Africa [2]. Early-stage HCC is usually treated with surgery, and in some cases liver transplantation is required. However, most patients present with unresectable advanced liver cancer, which requires local treatment or systemic therapy [3]. Unfortunately, systemic therapies for HCC are still limited and prognosis for advanced

HCC generally poor. Novel anti-HCC and anti-HBV-related HCC agents with novel targets or mechanisms are still urgently needed.

Ribonucleotide reductase (RR) catalyzes the conversion of ribonucleotides (NDPs) to deoxyribonucleotides (dNDPs), which are the building blocks for DNA synthesis and thus indispensable for cell proliferation. The holoenzyme of RR consists of large subunit M1 (RRM1) and small subunit M2 (RRM2) or RRM2B [4]. Pan-cancer expression profiling studies showed that the expression of RRM2 and RRM1 are upregulated in multi-types of cancers, and inhibition of RR activity has been proven to be an important anticancer strategy [5-7]. It has been shown previously that in HCC tissues RRM2 is upregulated compared with adjacent normal liver tissues [7, 8], and HBV which has no RR genes can activate RRM2 expression and RR enzyme activity in host liver cells thereby contributing to HBV DNA replication and HCC proliferation [9-11]. The high expression of RRM2 may be a poor prognostic factor for HCC and HBV-related HCC patients as well [12]. Therefore, RRM2 has been assumed to be a promising therapeutic target for HCC and HBV-related HCC treatment.

Several RRM2 inhibitors are currently clinically used for anticancer and antivirus therapy [5]. Hydroxyurea (HU), a representative RRM2 inhibitor, is used in clinical treatments for glioblastoma, chronic myelogenous leukemia, acute myelocytic leukemia, and sickle cell anemia alone or combined with other drugs for decades [13-16]. Recently, N-(4-(3,4-dihydroxyphenyl)-5-phenylthiazol-2-yl)-3,4-dihydroxybenzamide (COH29) was shown to possess higher RRM2 inhibitory activity and undergoes an anti-cancer phase I clinical trial [17]. We have also identified osalmid together with its derivative 4-Cyclopropyl-2-fluoro-N-(4-hydroxyphenyl) benzamide (YZ51) as potent inhibitors of RRM2 against HBV replication and HCC proliferation, respectively [11]. However, the target specificity, efficacy, and safety of these RRM2 inhibitors still need to be improved.

Pterostilbene (4-[(*E*)-2-(3,5-Dimethoxyphenyl) ethenyl]phenol) is known as a natural plant product and primarily found in blueberries and grapes [18]. It is a dimethylated derivative of resveratrol but has a much greater bioavailability compared with resveratrol [19]. It has been known that pterostilbene possesses multiple biological activity including antitumor effects [20]. In this study, using RRM2 3-dimentional structure-based computer-assisted screening of drug-like compound libraries, RR enzyme activity and cellular assays, as well as target and action mechanism validations, we for the first time identified pterostilbene as a potent inhibitor for RR activity and HBV DNA replication by interacting with RRM2. It simultaneously inhibited HCC proliferation and HBV replication potently. Mouse xenograft experiments showed that the compound effectively inhibited HCC cell growth with a relative low toxicity in vivo. Thus, the findings suggest a potential novel use of pterostilbine for treatment of HCC and especially HBV-related HCC.

Material and methods

Compounds

Pterostilbene (CAS Registry Number: 537-42-8) and COH29 (CAS Registry Number: 1190932-38-7) were bought from Topscience (Shanghai, China). Hydroxyurea (HU) was bought from Sigma-Aldrich (St. Louis, MO). Sorafenib was bought from Selleck (Shanghai, China). Lamivudine (2'-3'deoxy-3'-thiocytidine, 3TC) was bought from GlaxoSmithKline (Brentford, London).

Virtual screening

The crystal structure of RRM2 protein was retrieved from the protein data bank (PDB code: 3OLJ), and Glide5.5 (Schrödinger, LLC, New York, NY, USA) was used for docking. Remove the crystallographic water molecules in 3OLJ. Select F244, D271, R330 and E334 to define the center of the grid box.

Cell culture and transfection

Huh7 and Hep3B, the human hepatocarcinoma cell lines were purchased from the Cell Bank (Shanghai, China), HepG2, the human hepatocarcinoma cell line was purchased from the ATCC. HepG2.2.15, the human hepatocarcinoma cell line (contains 1.3-copy HBV genome), was purchased from the China Center for Type Culture Collection (CCTCC, Wuhan, China). The sorafenib-resistant HepG2 cells (HepG2-SR) and its counterpart wild-type HepG2 cells (HepG2-WT) were kindly provided by Dr. Xiujun

Cai [21]. Culture conditions and cell transfection method refer to the previous introduction [11].

RR activity measurements

The expression and purification method of RRM1 and RRM2 proteins refer to the previous introduction [22], A dCDP formation assay was used in RR activity measurement [22, 23]. The calculation of RR activity and inhibition analysis refer to our previous introduction [11].

Binding analysis using fluorescence quenching

To assay the binding between small compounds and proteins as described previously [24]. Pterostilbene was titrated into a solution of 1 μM RRM2 at 2.5-100 μM in two fold increments. The emission spectra were recorded over 290-400 nm using a fluorescence spectrophotometer. The K_D vaule was calculated as described previously [24].

Cancer cell growth inhibition assays

Cells were seeded into 96-well tissue culture plates at 5,000 cells per well. The next day, an equal volume of 2X drug-containing media was added. The cells were cultured for 3 additional days, and then cell growth was determined by MTT assays, and the results were measured at 490 nm.

Clonogenic assays

Cells were plated in 6-well plates at 10,000 cells per well, treated with a serial concentrations of pterostilbene for 14 days. Fix the colonies in methanol for 30 minutes and stain the colonies with 0.5% crystal violet for 30 minutes. The number of colonies (>50 cells) were scored using optical microscope. Colony formation rate was calculated as the number of colonies/10,000 × 100%.

Apoptosis analyses

Cells were plated in 6-well plates and treated with a series of concentrations of pterostilbene for 48 h. Then, stain the treated cells with V-FITC and PI for 30 minutes. Apoptosis was determined using Focusing Cytometer (FC500 MPL Beckman Coulter).

Determination of HBV DNA

HCC cells were seeded in 6-well plates. The next day, the cells were treated with a series concentrations of pterostilbene for 3 days, and then the culture media were changed with fresh pterostilbene and treated for another 3 days. After totally 6-day exposures, the cells were collected. As described previously [25], total DNA was purified from the cultured cells with the NucleoSpin DNA RapidLyse kit (Macherey Nagel, Germany). Measure the HBV DNA levels using Real-time polymerase chain reaction (Q-PCR). Sense and antisense primers were (fwd: GTTGCCCGTTTGTCCTCTAATTC, rev: GGAGGGATACATAGAGGTTCCTTGA). Q-PCR was performed with LightCycler 480 SYBR Green mix kit (Roche) using the 480Q-PCR System (Roche).

EdU incorporation assays

Cells were seeded in 96-well plates. The next day treat the cells with a serial concentrations of pterostilbene for 24 h, then asses the DNA synthesis by the Cell-Light EdU DNA cell proliferation kit (RiboBio Co., China). Images of the cells were captured with a fluorescence microscope.

Cell cycle analyses

Plate the cells in 6-well plates and treat the cells with pterostilbene for 24 h. Cells were fixed in 70% ethanol at -20°C over night. Wash the cells for two times in PBS and staine with RNase A and PI for 30 min. Focusing Cytometer (FC500 MPL Beckman Coulter) was used to determine the cell cycle distribution.

Immunoprecipitation coupled with HPLC/MS/MS analysis

The HepG2.2.15 cells were transfected with the expression vectors for FLAG-EV or FLAG-RRM2 [26]. Then the transfected cells were treated with or without pterostilbene for 24 h. Wash the cells for three times in PBS. The cell lysates were then analyzed by immunoprecipitation using anti-FLAG® M2 magnetic beads (Sigma-Aldrich). The eluted protein was verified by western blotting with FLAG antibody (CST). The pterostilbene bound to the eluted protein was measured using HPLC/MS/MS at Zhejiang University Analysis Center of Agrobiology and

Environmental Science. HPLC/MS/MS analyses were performed using an Agilent 6460 triple quadrupole mass spectrometer (Agilent Technologies, USA) equipped with an electrospray ionization (ESI) source, operated in the negative ion multiple-reaction monitoring (MRM) mode. Agilent Mass Hunter Workstation was used for data acquisition and processing [27].

Mouse tumor xenograft experiments

4-week old nude mice (nu/nu, male), brought from Shanghai SLAC Laboratory Animal Co., China) were subcutaneously injected with 1 × 10^7 HepG2 cells on their right flanks. After the tumors' size reached 100 mm^3 each mouse were treated with pterostilbene (50 or 100 mg/kg/day) dissolved in the solvent DMSO by intraperitoneal injection for 2 weeks. Tumor sizes were recorded by measurement of two perpendicular diameters of the tumors during the 2-week period by the formula 1/2 × $width^2$ × length. After 2 weeks harvest the tumors and weigh. The animal experiments were allowed in the ethical approval with the Laboratory Animals Welfare Ethics Review Committee of Zhejiang University (ZJU20170522).

Measurements of serum ALT and AST activity

After 2-week treatments, collect serum samples from each group of the mice. The ALT (Alanine aminotransferase) and AST (Aspartate aminotransferase) activities were measured at Zhejiang Chinese Medical University Laboratory Animal Research Center.

Histopathological examination

Fix the heart, liver, spleen, lung, and kidney tissues of mice in formalin, embed in paraffin, and cut into 3-5 μm sections, stain with hematoxylin and eosin (H&E), and examine by optical microscope at × 200 magnification.

Statistical analysis

Data were analyzed with GraphPad Prism 8.0.1, all values were expressed as the mean ± SD (standard deviation). The significance of the data was determined by the 2-tailed Student's t test. *P* values < 0.05 were considered significant.

Results

Pterostilbene inhibits RR enzyme activity by interacting with RRM2 protein

Using computer-aided screening of small molecule compounds, subsequent RR enzyme activity assays and fluorescence quenching analyses, we identified a compound, pterostilbene (**Figure 1A** and **1B**), as a RRM2 inhibitor. The concentration of pterostilbene inhibits the *in vitro* recombinant RR enzyme (RRM2 and RRM1 proteins) activity to 50% (IC_{50}) was 0.62±0.07 μM, which was 150-fold and 20-fold lower than that of HU (the representative RRM2 inhibitory drug) and COH29 (the known most potent RRM2 inhibitory drug presently in an anti-cancer phase I clinical trial), respectively (**Figure 1C** and **Table 1**).

The binding of pterostilbene to RRM2 protein was assessed using a fluorescence quenching assay [24] (**Figure 1D** and **1E**). To investigate whether pterostilbene bound to RRM2 in HCC cells, HepG2.2.15 cells were transfected with FLAG-EV or FLAG-RRM2 expression plasmids and followed by treated with or without pterostilbene. The cell lysates were then analyzed by immunoprecipitation-western blotting using FLAG antibody combined with HPLC/MS/MS detection. The results showed that pterostilbene bound to the RRM2 protein (**Figure 1F** and **1G**), but not to the irrelevant protein JMJD5 as a negative control in the HepG2.2.15 cells (Figure S1). The results indicated that pterostilbene inhibits RR enzyme activity by targeting RRM2 protein.

Pterostilbene exhibits an anti-proliferative effect on HCC cells and HBV genome-integrated HCC cells through inhibiting DNA synthesis

To determine the concentration dependence of the growth-inhibitory activity of pterostilbene, in comparison to the RRM2 inhibitors HU and COH29 and the first-line anti-HCC drug sorafenib, four established HCC cell lines were exposed to a full concentration range of each compound for 72 h, respectively (**Figure 2A**). As **Table 2** shown, the concentration of pterostilbene reduces these HCC cell viability to 50% (IC_{50}) were at 20 to 30 μM levels, much potent than HU, similar to COH29, and a few fold less effective than that of sorafenib. Notably, pterostilbene had a better inhibitory effect than HU

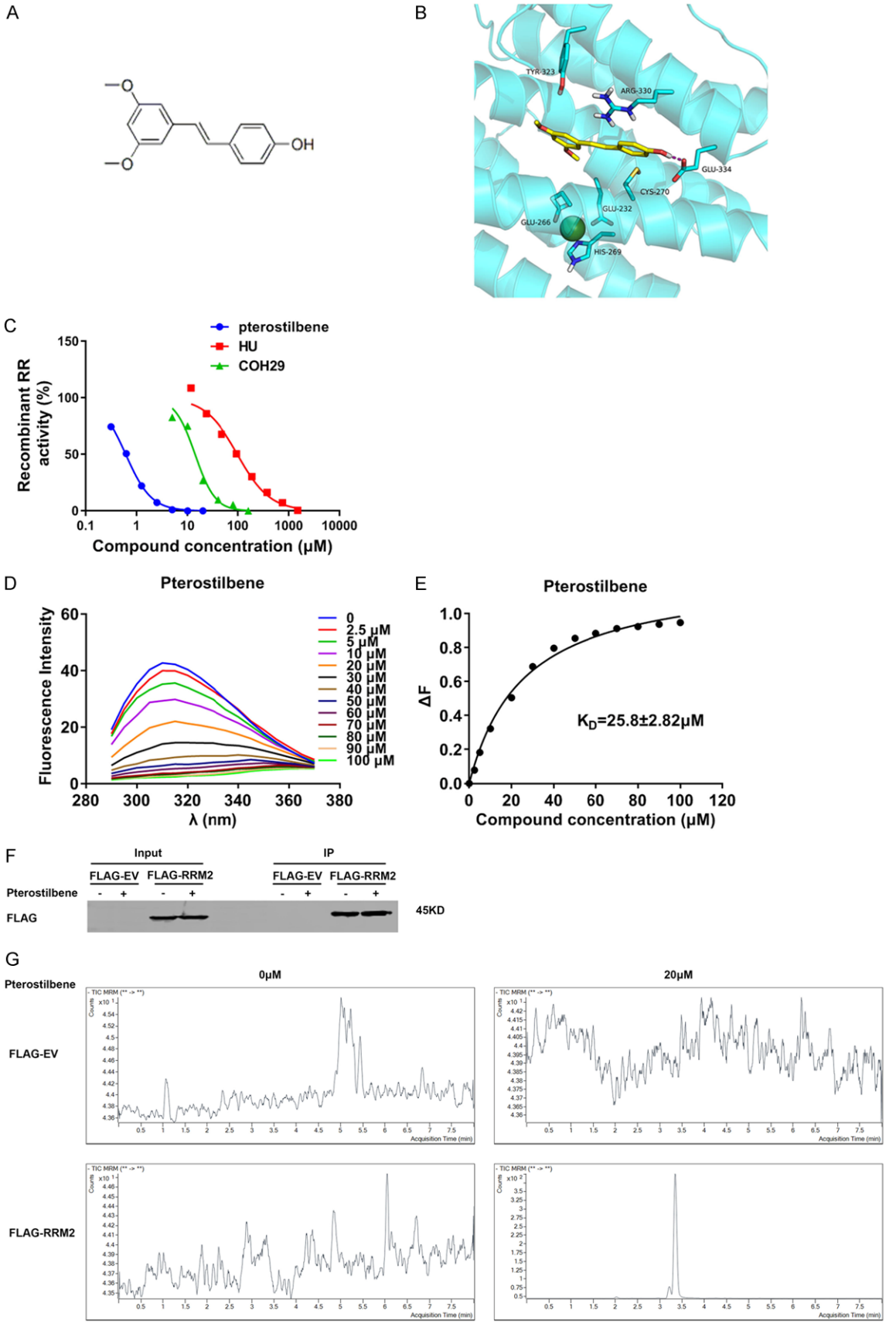

A
OH
B
TYR-323
ARG-330
GLU-334
CYS-270
GLU-232
GLU-266
HIS-269
C
pterostilbene
HU
COH29
Recombinant RR activity (%)
Compound concentration (μM)
D
Pterostilbene
Fluorescence Intensity
λ (nm)
0
2.5 μM
5 μM
10 μM
20 μM
30 μM
40 μM
50 μM
60 μM
70 μM
80 μM
90 μM
100 μM
E
Pterostilbene
ΔF
K_D=25.8±2.82μM
Compound concentration (μM)
F
Input
IP
FLAG-EV
FLAG-RRM2
Pterostilbene
FLAG
45KD
G
Pterostilbene
0μM
20μM
FLAG-EV
FLAG-RRM2
Acquisition Time (min)

Figure 1. Pterostilbene inhibits RR enzyme activity by targeting RRM2. A. Chemical structure of pterostilbene. B. Docking model showing the interaction between pterostilbene and key residues of RRM2 (PDB: 3OLJ). The protein structure is shown in blue cartoon. Hydrogen bond is shown in red dashed line. C. Sigmoidal dose-response curves and IC_{50} values of pterostilbene (0.3125 to 20 μM), COH29 (5 to 160 μM), and HU (11.71875 to 1500 μM) for inhibition of RR activity. D. Quenching of the tryptophan fluorescence of RRM2 by pterostilbene. Tryptophan fluorescence spectra of RRM2 (1 μM in PBS buffer) at a serial concentrations of pterostilbene (2.5-100 μM). E. Variation of the extent of fluorescence quenching [$(F_0-F)/F_0$, where F_0 and F are the fluorescence intensities of RRM2 at 325 nm in the absence and in the presence of pterostilbene, respectively]. F. HepG2.2.15 cells were transfected with FLAG-EV or FLAG-RRM2 expression plasmids, then treated with or without 20 μM pterostilbene for 24 h. The cell lysates were analyzed by immunoprecipitation using anti-FLAG magnetic beads followed by western blotting with FLAG antibody. G. The pterostilbene bounded to the RRM2 protein was detected by HPLC/MS/MS, the retention time for pterostilbene was 3.34 min. RR: Ribonucleotide reductase; RRM2: Ribonucleotide reductase small subunit M2; IC_{50}: The concentration of compound inhibits the in vitro recombinant RR enzyme (RRM2 and RRM1 proteins) activity to 50%; HU: Hydroxyurea; PBS: phosphate buffer saline. Results are presented as the mean ± SD of triplicate experiments, IP, immunoprecipitation.

Table 1. IC_{50} values of pterostilbene, HU, and COH29 for inhibition of RR activity

Compound	IC_{50} (μM)
Pterostilbene	0.62±0.07
HU	98.17±11.94
COH29	14.13±1.26

IC_{50}: The concentration of compound inhibits the in vitro recombinant RR enzyme (RRM2 and RRM1 proteins) activity to 50%; HU: Hydroxyurea.

and COH29 on the HBV-genome integrated HCC cell line HepG2.2.15. Colony formation assays and apoptosis analyses also showed that pterostilbene potently inhibited the proliferation and induced the apoptosis of HepG2.2.15 cells in a dose-dependent manner (**Figure 2B-E**).

To further investigate the cellular mechanism of action of pterostilbene, HepG2.2.15 cells were treated with different concentrations of the compound for 24 h. EdU incorporation and flow cytometry analyses showed that the treatments dose-dependently inhibited DNA synthesis (**Figure 2F** and **2G**) and induced cell cycle S-phase arrest (**Figure 2H** and **2I**), respectively, in HepG2.2.15 cells. The results were well in accordance with the consequence of RR function inhibition.

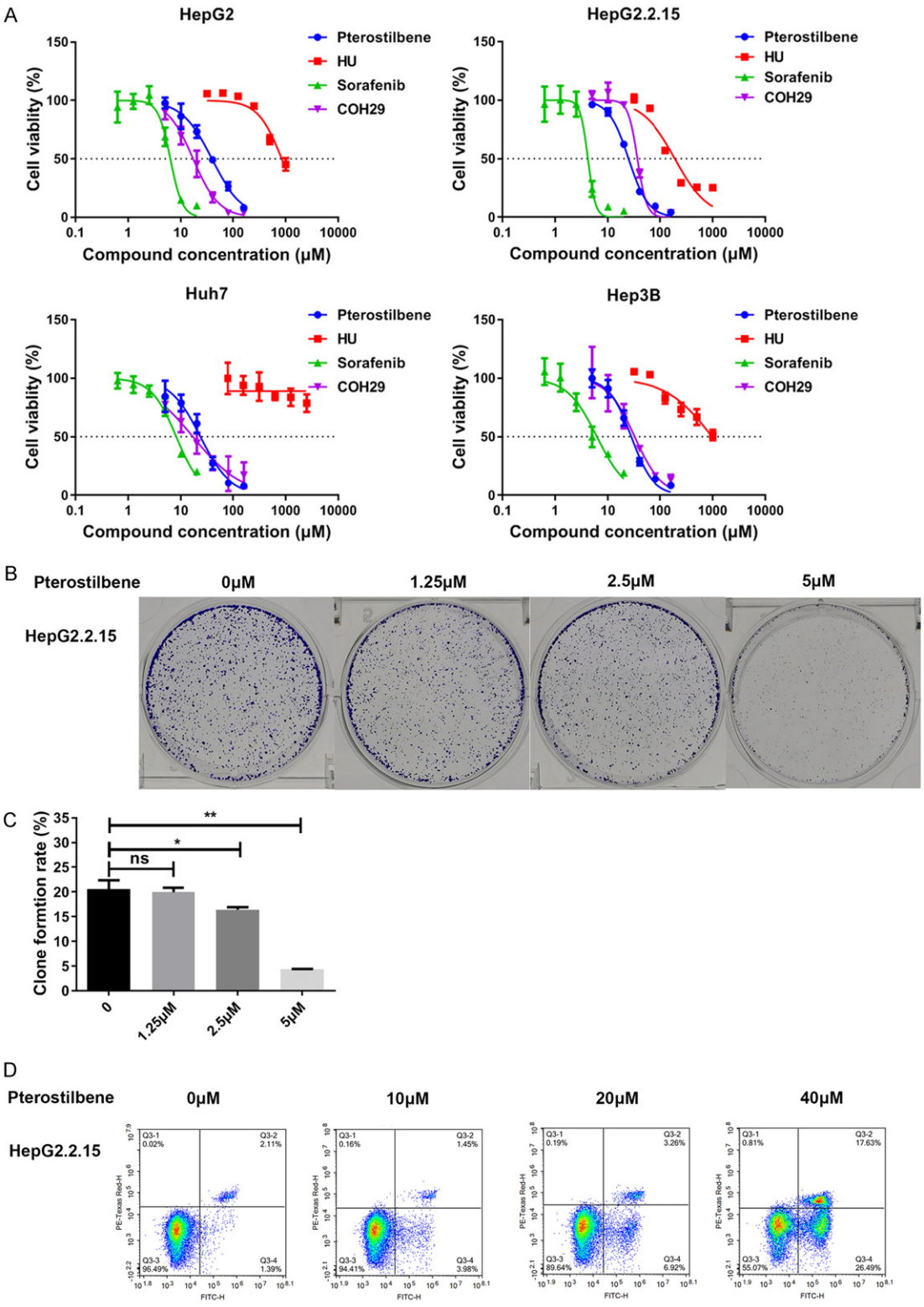
Pterostilbene possesses a similar inhibitory effect against sorafenib-resistant and wild-type HCC cells

Drug resistance is a major drawback of sorafenib in clinical HCC treatment. A strain of sorafenib-resistant HepG2 cells (HepG2-SR) and its parental wild-type HepG2 cells (HepG2-WT) [21], were used to evaluate the ability of pterostilbene to overcome sorafenib resistance. As determined by MTT assays (**Figure 3** and **Table 3**), pterostilbene displayed a similar anti-proliferative effect against both HepG2-SR and HepG2-WT cells, in comparison with the resistance of sorafenib in the cells.

Pterostilbene inhibits HBV DNA replication in HBV genome-integrated and transfected HCC cells and overcomes lamivudine resistance

In order to determine whether pterostilbene can inhibit HBV replication, we assessed its inhibitory effects on HBV DNA replication in the HBV-genome integrated HCC cell line HepG2.2.15 and the HCC cell line HepG2 transfected with 1.3-copy HBV DNA. The results showed that pterostilbene significantly inhibited HBV DNA replication in a dose-dependent manner after 6-day treatments (**Figure 4A**, **4B** and **Table 4**). The EC_{50}s for inhibiting HBV DNA replication by 50% as determined by Q-PCR measurements were about 4 fold lower than the IC_{50} for the viability of HepG2.2.15 cells as determined by MTT assays, indicating that HBV DNA replication is more sensitive to pterostilbene than the host HepG2.2.15 cell proliferation.

Lamivudine (3TC) is the first nucleoside analogue drug for hepatitis B treatment by inhibiting HBV DNA polymerase. To further evaluate whether pterostilbene could overcome 3TC-resistance, HepG2 cells were transfected with the HBV DNA plasmids (wild-type or 3TC resistance mutant) [11]. Then, the HepG2 cells were treated with a series concentrations of pterostilbene or 3TC for 6 days, and then the amount of HBV DNA in the cells was evaluated by Q-PCR assays. As shown in **Figure 4C**, **4D** and **Table 5**,


A
HepG2
HepG2.2.15
Huh7
Hep3B
Pterostilbene
HU
Sorafenib
COH29
Cell viability (%)
Cell viablity (%)
Compound concentration (μM)
B
Pterostilbene
0μM
1.25μM
2.5μM
5μM
HepG2.2.15
C
Clone formtion rate (%)
ns
*
**
0
1.25μM
2.5μM
5μM
D
Pterostilbene
0μM
10μM
20μM
40μM
HepG2.2.15
PE-Texas Red-H
FITC-H
Q3-1 0.02%
Q3-2 2.11%
Q3-3 96.49%
Q3-4 1.39%
Q3-1 0.16%
Q3-2 1.45%
Q3-3 94.41%
Q3-4 3.98%
Q3-1 0.19%
Q3-2 3.26%
Q3-3 89.64%
Q3-4 6.92%
Q3-1 0.81%
Q3-2 17.63%
Q3-3 55.07%
Q3-4 26.49%

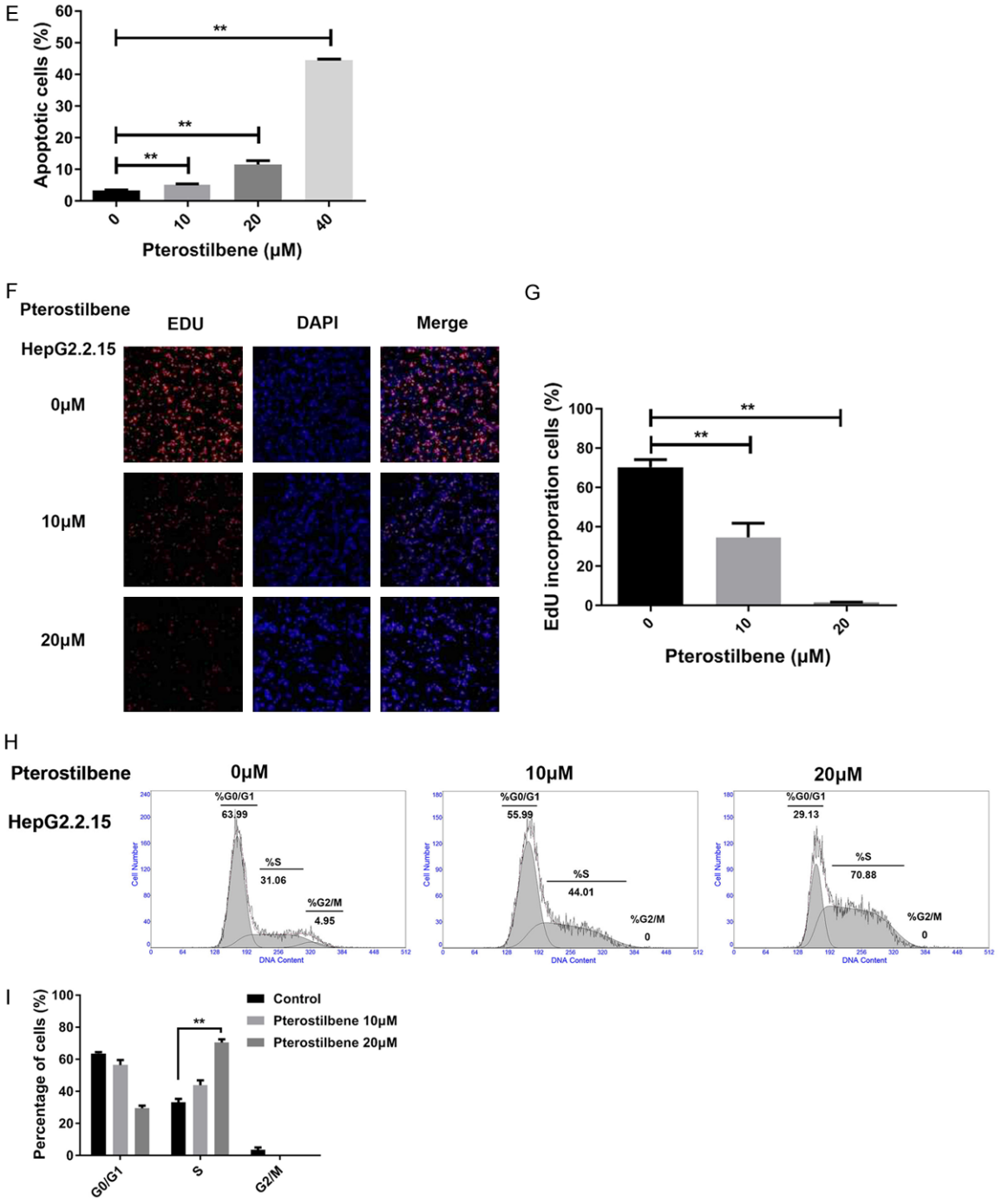


Figure 2. Pterostilbene inhibits HCC proliferation through inhibiting DNA synthesis. A. The anti-proliferation activities of pterostilbene, HU, sorafenib and COH29 for HCC cell lines (HepG2, HepG2.2.15, Huh7, and Hep3B). Treat the cells with compound for 72 h, and quantitate the cell viability by MTT assays. B, C. Treat the HepG2.2.15 cells with pterostilbene (1.25, 2.5, and 5 μM) for 14 days, and the colony formation rates were measured. D, E. Treat the HepG2.2.15 cells with pterostilbene (10, 20, and 40 μM) for 48 h, and the apoptosis rates were determined. F, G. Treat the HepG2.2.15 cells with pterostilbene for 24 h, and then the newly synthesized DNA amounts were measured by the EdU incorporation assays. Red fluorescence: the amount of newly synthesized DNA, blue fluorescence: the total number of nuclei stained by DAPI. H, I. Treat the HepG2.2.15 cells with pterostilbene for 24 h, and then determin the cell cycle distribution using flow cytometry. HCC: Hepatocellular carcinoma; DNA: DeoxyriboNucleic Acid; HU: Hydroxyurea; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; EdU: 5-ethynyl-2'-deoxyuridine; DAPI: 4',6-Diamidino-2-phenylindole,dihydrochloride. Results are presented as the mean ± SD of triplicate experiments (Student t test, *P < 0.05 and **P < 0.005 vs. the negative control group, ns: no significant difference).

Table 2. Inhibitory effects of Pterostilbene, HU, Sorafenib and COH29 on cell viability in HCC cells

Compound	Treatment time (days)	IC_{50} for cell vability (μM)			
		HepG2	HepG2.2.15	Huh7	Hep3B
Pterostilbene	3	39.06±4.15	24.59±1.18	23.60±3.71	27.84±2.83
HU	3	844.10±110.90	190.20±40.70	>2500	>1000
Sorafenib	3	6.33±0.73	4.19±0.58	8.17±0.91	6.29±1.25
COH29	3	17.19±1.94	36.26±2.31	17.37±4.30	32.90±7.17

IC_{50}: The concentration of compound reduces these HCC cell viability to 50%; HU: Hydroxyurea.

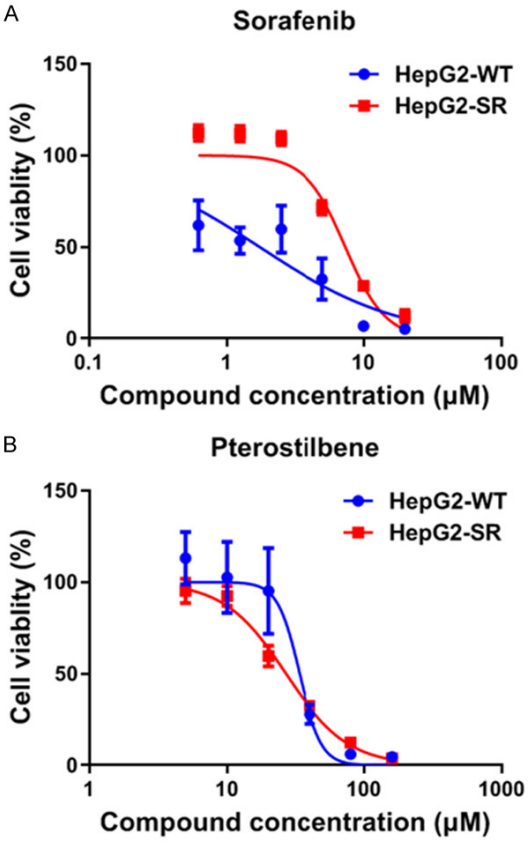


Figure 3. Inhibitory effects of pterostilbene on the proliferation of the wild-type and sorafenib-resistant HCC cells. (A) The wild-type (HepG2-WT) and (B) sorafenib-resistant (HepG2-SR) HepG2 cells were treated with a series concentrations of sorafenib or pterostilbene for 72 h, and the cell viability was quantitated by MTT assays. HCC: Hepatocellular carcinoma; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. Results are presented as the mean ± SD of triplicate experiments.

the effectiveness of 3TC in inhibiting HBV DNA replication of the mutant strain was reduced about 20-fold. Whereas, pterostilbene strongly inhibited the HBV DNA replication of both the wild-type and 3TC-resistant mutant strains, even more potent for the 3TC-resistant HBV

Table 3. Inhibitory effects of Sorafenib and Pterostilbene on cell viability in HepG2-WT and HepG2-SR cells

Compound	IC_{50} for cell vability (μM)	
	HepG2-WT	HepG2-SR
Sorafenib	1.88±0.72	7.47±1.15
Pterostilbene	34.28±6.26	26.92±2.38

HepG2-WT: wild-type HepG2; HepG2-SR: sorafenib-resistant HepG2; IC_{50}: The concentration of compound reduces these HCC cell viability to 50%.

strain. In addition, the EC_{50}s for inhibiting HBV DNA replication were about 40-fold lower than the IC_{50} for the viability of the host HepG2 cells transfected with HBV DNA, also supporting that HBV DNA replication is much more sensitive to pterostilbene than the host HCC cell proliferation.

The inhibitory effects of pterostilbene on RR enzyme activity can be reversed by the addition of dNTP precursors

RR is the unique enzyme to produce dNTPs as substrates for DNA synthesis, addition of the products can counteract the effects of RR inhibition [28]. As dNTPs penetrate cell membranes poorly, a mixture of their precursors (dNs, including thymidine, deoxyadenosine, deoxyguanosine, and deoxycytidine) was used instead. As shown in **Figure 5A**, the addition of 100 μM dNs reversed the proliferation inhibition of HepG2 and HepG2.2.15 cells by 20 μM pterostilbene. The inhibition of HBV DNA replication in HepG2.2.15 cells was also partially reversed by the addition of 100 μM dNs (**Figure 5B**). Furthermore, the cell cycle s-phase arrest caused by pterostilbene was reversed as well (**Figure 5C**). The results showed that the inhibitory effects of pterostilbene can be reversed by addition of dNs, suggesting that RR is the acting target of pterostilbene in the treated cells.

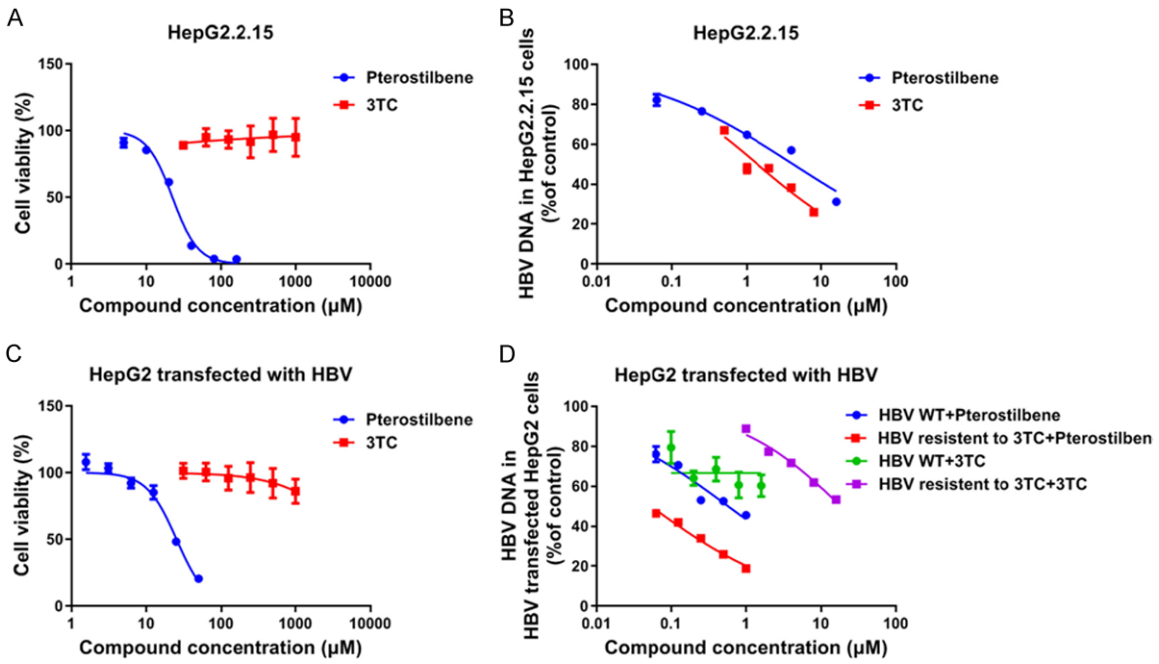


Figure 4. Inhibitory effects of pterostilbene on HBV replication. A, B. Treat the HepG2.2.15 cells with pterostilbene (0.03125 to 16 μM) or 3TC (0.5 to 8 μM) for 6 days, and the inhibitory effects of pterostilbene or 3TC on intracellular HBV DNA replication and cell viability were detected by Q-PCR and MTT assays, respectively. C, D. HepG2 cells were transfected with the wild-type or 3TC-resistant-mutant 1.3-copy HBV DNA plasmid, and then exposed to pterostilbene or 3TC for 6 days. The cell viability and HBV DNA amounts in the cells were quantified by MTT and Q-PCR assays, respectively. HBV: Hepatitis B virus; DNA: DeoxyriboNucleic Acid; 3TC: Lamivudine; Q-PCR: Real-time polymerase chain reaction; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; EC_{50}: the concentration at which the compound inhibits 50% HBV DNA replication. Results are presented as the mean ± SD of triplicate experiments.

Table 4. Inhibitory effects of Pterostilbene and 3TC on HBV replication and cell viability in HepG2.2.15 cells

Compound	EC_{50} (μM)	IC_{50} (μM)
Pterostilbene	5.65±1.44	22.37±1.68
3TC	1.42±0.28	>1000

3TC: Lamivudine; EC_{50}: The concentration of compound reduces HBV DNA replication to 50%.

Table 5. Inhibitory effects of Pterostilbene and 3TC on HBV replication and cell viability in HBV transfected HepG2 cells

Compound	EC_{50} (μM)		IC_{50} (μM)
	WT	Mut	
Pterostilbene	0.6±0.16	0.039±0.007	25.5±2.49
3TC	≈0.8	17.42±3.09	>1000

3TC: Lamivudine; EC_{50}: The concentration of compound reduces HBV DNA replication to 50%; IC_{50}: The concentration of compound reduces these HCC cell viability to 50%; WT: wild-type; Mut: 3TC-resistant-mutant.

Pterostilbine potently inhibits HCC cell growth in tumor xenograft mice with a low toxicity under the treatment doses

To test the effect of pterostilbine on tumour growth in vivo, nude mice were injected with HepG2 cells and treated with 50 or 100 mg/kg/day of pterostilbine for 14 days. The results showed that the pterostilbine treatments significantly decreased HCC cell growth in the nude mice compared with the control group (**Figure 6A-C**). At the same time, neither mouse body weights nor ALT (Aspartate aminotransferase) and AST (Aspartate aminotransferase) levels were significantly changed between the pterostilbine-treated groups and the solvent control group (**Figure 6D** and **6E**). Moreover, no notable intergroup differences were observed in the important organs of the treated mice, including the heart, liver, spleen, lung, and kidney (**Figure 6F**). The results indicated a relative

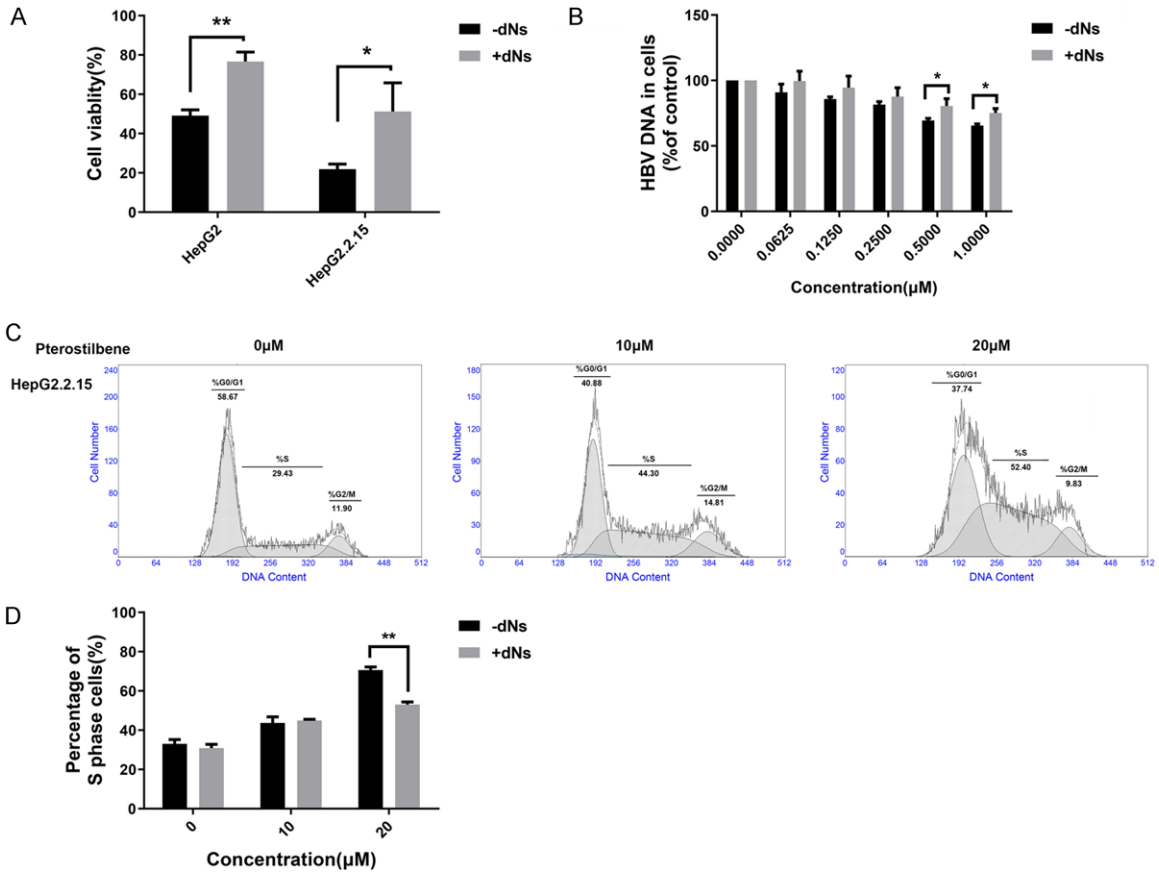


Figure 5. dNs can reverse the effects of RR inhibition by pterostilbene. A. HepG2 or HepG2.2.15 cells were treated with 100 μM dNs (including thymidine, deoxyadenosine, deoxyguanosine, and deoxycytidine) for 24 h, then further treated by adding a series concentrations of pterostilbene for 72 h, and the cell viability was quantitated by MTT assays. B. Treat the HepG2.2.15 cells with pterostilbene together with 100 μM dNs for 6 days, and the HBV DNA in the cells were detected by Q-PCR measurements. C, D. Treat the HepG2.2.15 cells with pterostilbene at 10 or 20 μM together with 100 μM dNs for 24 h, then the cell-cycle distributions were analyzed using Focusing Cytometer. MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HBV: Hepatitis B virus; DNA: DeoxyriboNucleic Acid; Q-PCR: Real-time polymerase chain reaction. Results are presented as the mean ± SD of triplicate experiments. *$P < 0.05$, **$P < 0.005$ vs no dNs treated cells by a two-tailed Student's t test.

low in vivo toxicity of pterostilbine at the effective doses used in this study.

Discussion

RRM2 expression has been confirmed significantly up-regulated in HCC cells, providing sufficient dNTPs to favor the malignant proliferation [6, 8, 29]. HBV has no RR genes and HBx can activate RRM2 expression in the host liver cells for the viral DNA replication [10]. HBV infection is a causative factor of HCC and associated with HCC development and prognosis. We have previously demonstrated that RR is essential for HBV genomic DNA and cccDNA synthesis in HCC cells, and RR enzyme activity is up-regulated in the liver tissues of HBV genome-transgenic mice [11]. These findings suggest that RR, HBV, and HCC may form a mutual-promoting vicious triangle during HBV-related HCC development.

In this study, for the first time pterostilbene was identified as a novel RRM2 inhibitor (**Figure 1**). RR enzyme assays showed that the compound possessed about 20-fold and 200-fold higher activity for RR inhibition than that of COH29 (the most effective RRM2 inhibitory drug in an anti-cancer phase I clinical trial) and HU (the classical RRM2 inhibitory drug), respectively. The higher RR inhibitory activity may indicate a higher specificity for RRM2 and a lower off-target effect of pterostilbene. Pterostilbene potently inhibited HCC proliferation and HBV

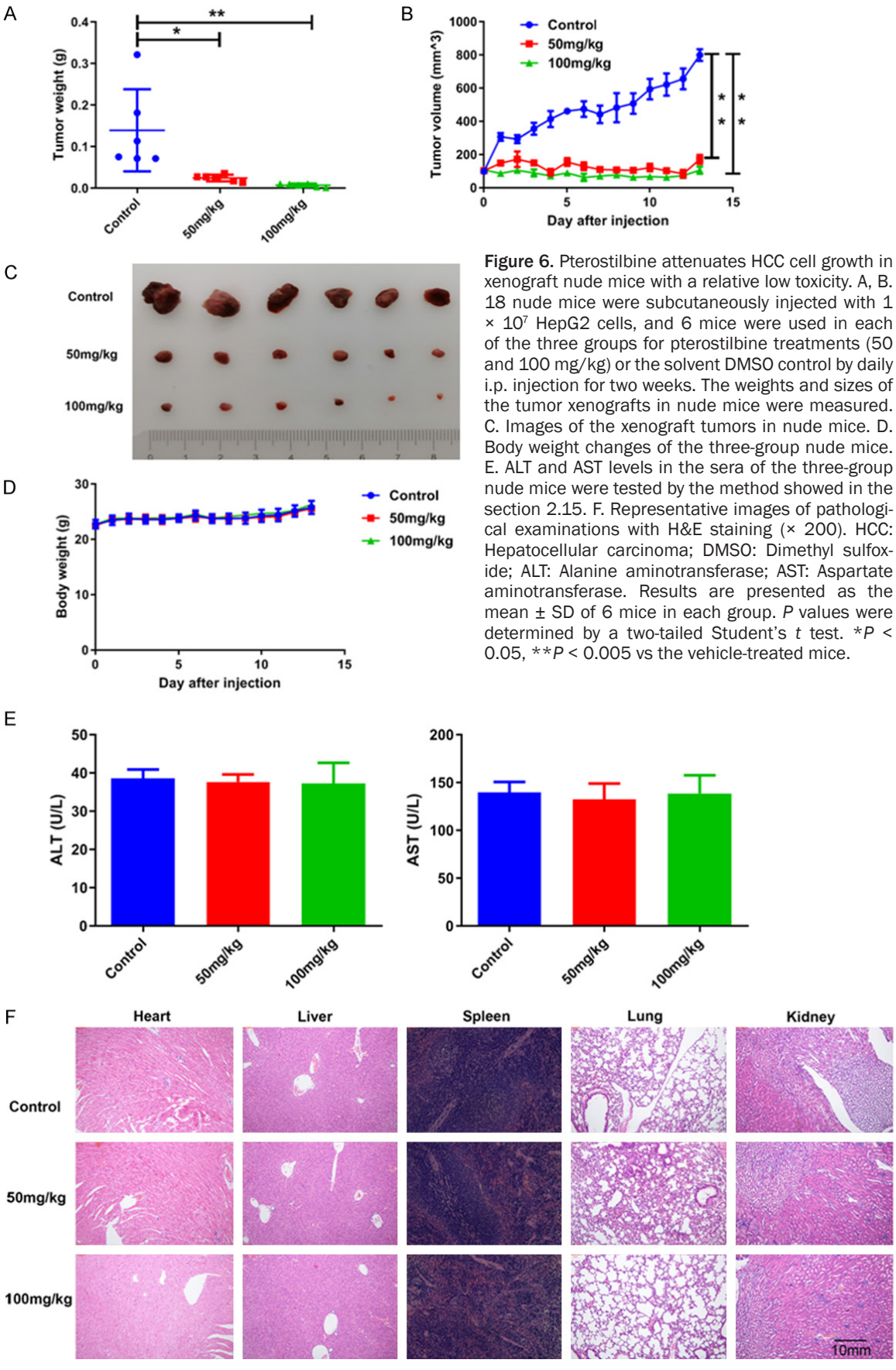


Figure 6. Pterostilbine attenuates HCC cell growth in xenograft nude mice with a relative low toxicity. A, B. 18 nude mice were subcutaneously injected with 1 × 10^7 HepG2 cells, and 6 mice were used in each of the three groups for pterostilbine treatments (50 and 100 mg/kg) or the solvent DMSO control by daily i.p. injection for two weeks. The weights and sizes of the tumor xenografts in nude mice were measured. C. Images of the xenograft tumors in nude mice. D. Body weight changes of the three-group nude mice. E. ALT and AST levels in the sera of the three-group nude mice were tested by the method showed in the section 2.15. F. Representative images of pathological examinations with H&E staining (× 200). HCC: Hepatocellular carcinoma; DMSO: Dimethyl sulfoxide; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase. Results are presented as the mean ± SD of 6 mice in each group. *P* values were determined by a two-tailed Student's *t* test. **P* < 0.05, ***P* < 0.005 vs the vehicle-treated mice.

DNA replication in HCC cells (**Figures 2** and **4**), while the both inhibitory effects were significantly reversed by the addition of dNTP precursors, indicating that RR was the intracellular action target of pterostilbine (**Figures 1F**, **1G** and **5**).

Pterostilbine inhibited *in vitro* HCC proliferation with a MTT IC_{50} of about 20-40 μM, resulted from the inhibition of RR, the rate-limiting enzyme in the synthesis of dNTPs, leading to DNA synthesis blocking, cell cycle arrest, and finally apoptosis (**Figure 2**). The inhibitory potency was similar to COH29 but a little lower than sorafenib (**Figure 2**). Since 2007, sorafenib, a multi-kinase (serine, threonine, and tyrosine kinases) inhibitor, has been used as a clinical first-line targeted drug for HCC systemic therapy, but the overall survival rate is limited and the treated patients frequently developed resistance to sorafenib. Our data demonstrated that pterostilbene can overcome the sorafenib-resistance of HCC cells (**Figure 3**). Tumor xenograft mouse model assays showed that pterostilbine markedly inhibited HCC tumor growth with a relatively low toxicity under therapeutic doses *in vivo* (**Figure 6**), suggesting a potential use of the compound for HCC treatment, especially for sorafenib-resistant HCC.

On the other hand, pterostilbene significantly inhibited HBV DNA replication in both the HBV genome-integrated HCC cell line HepG2.2.15 and the HCC cell line HepG2 newly transfected with HBV DNA (**Figure 4**). The EC_{50} values of the compound for inhibiting HBV DNA replication were about 4-fold and 40-fold lower than its MTT IC_{50} values for inhibiting the host HCC proliferation in the two cell lines, respectively. The results suggested that the inhibition of HBV replication did not result from the death of the host cells. Further, HBV replicates quickly in the infected liver cells, consuming a large amount of dNTPs for its genome synthesis, which may explain why HBV replication was more vulnerable to RR inhibition by pterostilbene than its host HCC proliferation [9]. Interestingly, pterostilbene more potently inhibited the HBV-genome integrated HCC cell line HepG2.2.15 than HU and COH29 (**Figure 2**), possibly due to its higher specificity for RRM2 inhibition. Also importantly, while the HBV replication inhibitory activity of pterostilbene was close to that of lamivudine, it effectively overcame the resistance to the anti-HBV drug (**Figure 4**), a recurrent clinical problem during the long term anti-HBV treatment. The target and action mechanism of pterostilbene are different from lamivudine and sorafenib, which may explain its efficacy in overcoming the resistance to the two drugs.

In summary, this research shows that inhibition of RR has the double effects against both HBV replication and HCC proliferation. Pterostilbene is a novel potent and specific RR inhibitor through interacting with RRM2 protein. The compound possesses much higher activity against *in vitro* RR enzyme activity and *in vivo* HCC xenograft growth than current RRM2 inhibitory drugs. It can significantly simultaneously inhibit both HCC proliferation and HBV replication by RR inhibition with the potencies comparable to the anti-HCC drug sorafenib and the anti-HBV drug lamivudine, respectively. Furthermore, the compound can overcome the frequent drug-resistant problems with sorafenib and lamivudine. So far, no single drug has been reported to possess both activities against HBV and HCC. Thus, pterostilbene, as an available natural product or synthesized compound, or through further optimization, may prove to be a potential new drug for treatment of the HCC and especially HBV-related HCC in the future.

Acknowledgements

This work was supported by National Natural Science Foundation of China (81372138, 81972270, 81572384, and 81771518), National Science and Technology Major Project of China (2018ZX10302206-006-007), and National Key R&D Program of China (2016YFC1303401).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jimin Shao, Department of Pathology & Pathophysiology, and Cancer Institute of The Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China. Tel: +86-13732221892; Fax: +86-0571-88208209; E-mail: shaojimin@zju.edu.cn; Weiliang Zhu, Drug Discovery and Design Center, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 555 Zuchongzhi Road, Zhangjiang Hi-Tech Park, Pudong, Shanghai 201203, China. Tel: +86-13681867996; Fax: +86-21-50807088; E-mail: wlzhu@simm.ac.cn

References

[1] Siegel RL, Miller KD and Jemal A. Cancer statistics, 2020. CA Cancer J Clin 2020; 70: 7-30.

[2] de Martel C, Maucort-Boulch D, Plummer M and Franceschi S. World-wide relative contribution of hepatitis B and C viruses in hepatocellular carcinoma. Hepatology 2015; 62: 1190-1200.

[3] Benson AB, D'Angelica MI, Abbott DE, Abrams TA, Alberts SR, Anaya DA, Anders R, Are C, Brown D, Chang DT, Cloyd J, Covey AM, Hawkins W, Iyer R, Jacob R, Karachristos A, Kelley RK, Kim R, Palta M, Park JO, Sahai V, Schefter T, Sicklick JK, Singh G, Sohal D, Stein S, Tian GG, Vauthey JN, Venook AP, Hammond LJ and Darlow SD. NCCN guidelines insights: hepatobiliary cancers, version 2.2019. J Natl Compr Canc Netw 2019; 17: 302-310.

[4] Kang G, Taguchi AT, Stubbe J and Drennan CL. Structure of a trapped radical transfer pathway within a ribonucleotide reductase holocomplex. Science 2020; 368: 424.

[5] Shao J, Liu X, Zhu L and Yen Y. Targeting ribonucleotide reductase for cancer therapy. Expert Opin Ther Targets 2013; 17: 1423-1437.

[6] Aye Y, Li M, Long MJ and Weiss RS. Ribonucleotide reductase and cancer: biological mechanisms and targeted therapies. Oncogene 2015; 34: 2011-2021.

[7] Ding Y, Zhong T, Wang M, Xiang X, Ren G, Jia Z, Lin Q, Liu Q, Dong J, Li L, Li X, Jiang H, Zhu L, Li H, Shen D, Teng L, Li C and Shao J. Integrative analysis reveals across-cancer expression patterns and clinical relevance of ribonucleotide reductase in human cancers. Front Oncol 2019; 9: 956.

[8] Wang L, Huang J and Jiang M. RRM2 computational phosphoprotein network construction and analysis between no-tumor hepatitis/cirrhotic liver tissues and human hepatocellular carcinoma (HCC). Cell Physiol Biochem 2010; 26: 303-310.

[9] Gearhart TL and Bouchard MJ. Replication of the hepatitis B virus requires a calcium-dependent HBx-induced G1 phase arrest of hepatocytes. Virology 2010; 407: 14-25.

[10] Cohen D, Adamovich Y, Reuven N and Shaul Y. Hepatitis B virus activates deoxynucleotide synthesis in nondividing hepatocytes by targeting the R2 gene. Hepatology 2010; 51: 1538-1546.

[11] Liu X, Xu Z, Hou C, Wang M, Chen X, Lin Q, Song R, Lou M, Zhu L, Qiu Y, Chen Z, Yang C, Zhu W and Shao J. Inhibition of hepatitis B virus replication by targeting ribonucleotide reductase M2 protein. Biochem Pharmacol 2016; 103: 118-128.

[12] Xu J, Zhang J, Shan F, Wen J and Wang Y. SSTR5-AS1 functions as a ceRNA to regulate CA2 by sponging miR-15b-5p for the development and prognosis of HBV-related hepatocellular carcinoma. Mol Med Rep 2019; 20: 5021-5031.

[13] Levin VA. The place of hydroxyurea in the treatment of primary brain tumors. Semin Oncol 1992; 19: 34-39.

[14] Hehlmann R, Heimpel H, Hasford J, Kolb HJ, Pralle H, Hossfeld DK, Queisser W, Löffler H, Heinze B, Georgii A, et al. Randomized comparison of busulfan and hydroxyurea in chronic myelogenous leukemia: prolongation of survival by hydroxyurea. The German CML Study Group. Blood 1993; 82: 398-407.

[15] Sterkers Y, Preudhomme C, Laï JL, Demory JL, Caulier MT, Wattel E, Bordessoule D, Bauters F and Fenaux P. Acute myeloid leukemia and myelodysplastic syndromes following essential thrombocythemia treated with hydroxyurea: high proportion of cases with 17p deletion. Blood 1998; 91: 616-622.

[16] Platt OS. Hydroxyurea for the treatment of sickle cell anemia. N Engl J Med 2008; 358: 1362-1369.

[17] Zhou B, Su L, Hu S, Hu W, Yip ML, Wu J, Gaur S, Smith DL, Yuan YC, Synold TW, Horne D and Yen Y. A small-molecule blocking ribonucleotide reductase holoenzyme formation inhibits cancer cell growth and overcomes drug resistance. Cancer Res 2013; 73: 6484-6493.

[18] Rimando AM, Kalt W, Magee JB, Dewey J and Ballington JR. Resveratrol, pterostilbene, and piceatannol in vaccinium berries. J Agric Food Chem 2004; 52: 4713-4719.

[19] Lin HS, Yue BD and Ho PC. Determination of pterostilbene in rat plasma by a simple HPLC-UV method and its application in pre-clinical pharmacokinetic study. Biomed Chromatogr 2009; 23: 1308-1315.

[20] Ma Z, Zhang X, Xu L, Liu D, Di S, Li W, Zhang J, Zhang H, Li X, Han J and Yan X. Pterostilbene: mechanisms of its action as oncostatic agent in cell models and in vivo studies. Pharmacol Res 2019; 145: 104265.

[21] Xu J, Wan Z, Tang M, Lin Z, Jiang S, Ji L, Gorshkov K, Mao Q, Xia S, Cen D, Zheng J, Liang X and Cai X. N6-methyladenosine-modified CircRNA-SORE sustains sorafenib resistance in hepatocellular carcinoma by regulating β-catenin signaling. Molecular Cancer 2020; 19: 163.

[22] Zhu L, Zhou B, Chen X, Jiang H, Shao J and Yen Y. Inhibitory mechanisms of heterocyclic carboxaldehyde thiosemicabazones for two forms of human ribonucleotide reductase. Biochem Pharmacol 2009; 78: 1178-1185.

[23] Shao J, Zhou B, Zhu L, Bilio AJ, Su L, Yuan YC, Ren S, Lien EJ, Shih J and Yen Y. Determination of the potency and subunit-selectivity of ribonucleotide reductase inhibitors with a recombinant-holoenzyme-based in vitro assay. Biochem Pharmacol 2005; 69: 627-634.

[24] Ahmad MF, Huff SE, Pink J, Alam I, Zhang A, Perry K, Harris ME, Misko T, Porwal SK, Oleinick NL, Miyagi M, Viswanathan R and Dealwis CG. Identification of Non-nucleoside Human Ribonucleotide Reductase Modulators. J Med Chem 2015; 58: 9498-9509.

[25] Lucifora J, Xia Y, Reisinger F, Zhang K, Stadler D, Cheng X, Sprinzl MF, Koppensteiner H, Makowska Z, Volz T, Remouchamps C, Chou WM, Thasler WE, Hüser N, Durantel D, Liang TJ, Münk C, Heim MH, Browning JL, Dejardin E, Dandri M, Schindler M, Heikenwalder M and Protzer U. Specific and nonhepatotoxic degradation of nuclear hepatitis B virus cccDNA. Science 2014; 343: 1221-1228.

[26] Chen X, Xu Z, Zhang L, Liu H, Liu X, Lou M, Zhu L, Huang B, Yang CG, Zhu W and Shao J. The conserved Lys-95 charged residue cluster is critical for the homodimerization and enzyme activity of human ribonucleotide reductase small subunit M2. J Biol Chem 2014; 289: 909-920.

[27] Luo L, Shen L, Sun F and Ma Z. Immunoprecipitation coupled with HPLC-MS/MS to discover the aromatase ligands from Glycyrrhiza uralensis. Food Chemistry 2013; 138: 315-320.

[28] Crona M, Codó P, Jonna VR, Hofer A, Fernandes AP and Tholander F. A ribonucleotide reductase inhibitor with deoxyribonucleoside-reversible cytotoxicity. Mol Oncol 2016; 10: 1375-1386.

[29] Morikawa T, Hino R, Uozaki H, Maeda D, Ushiku T, Shinozaki A, Sakatani T and Fukayama M. Expression of ribonucleotide reductase M2 subunit in gastric cancer and effects of RRM2 inhibition in vitro. Hum Pathol 2010; 41: 1742-1748.

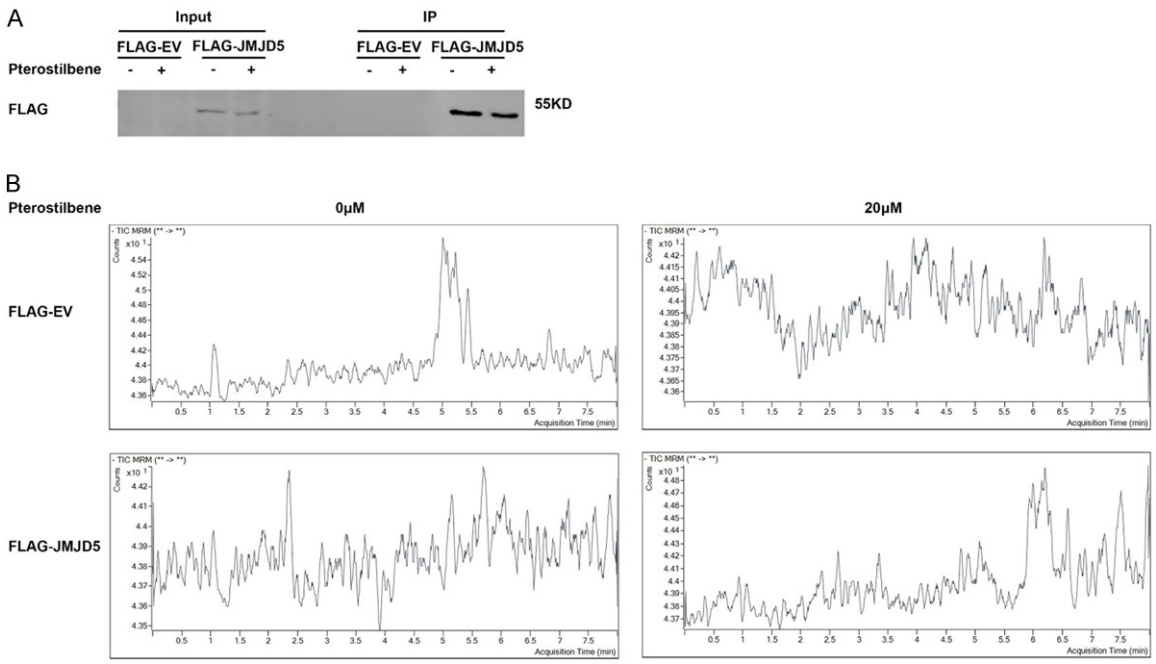


Figure S1. A. HepG2.2.15 cells were transfected with FLAG-EV or FLAG-JMJD5 expression plasmids, then the cells were treated with or without 20 μM pterostilbene for 24 h. The cell lysates were analyzed by immunoprecipitation using anti-FLAG magnetic beads followed by western blotting with FLAG antibody. B. The pterostilbene bounded to the eluted protein was detected by HPLC/MS/MS, retention time for pterostilbene was 3.34 min. IP, immunoprecipitation.