Dihydrotanshinone I inhibits hepatocellular carcinoma cells proliferation through DNA damage and EGFR pathway (#77039)

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Dihydrotanshinone I inhibits hepatocellular carcinoma cells proliferation through DNA damage and EGFR pathway

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Background: Hepatocellular carcinoma (HCC) incidence and mortality are escalating globally. Dihydrotanshinone I, a natural product isolated from *Salvia miltiorrhiza Bunge,* attracted extensive attention for its anti-cancer proliferation effect in recent years. **Methods:** The proliferation of Huh-7 and HepG2 hepatoma cells was evaluated using the MTT and clone formation assays. An immunofluorescence (IF) experiment of 53BP1 and a flow cytometry analysis were used to detect DNA damage and cell apoptosis, respectively. Moreover, network pharmacology analysis was applied to study the possible therapeutic targets and pathway of Dihydrotanshinone I. **Results:** Our results showed that Dihydrotanshinone I effectively inhibited the proliferation of Huh-7 and HepG2 hepatoma cells. Moreover, Dihydrotanshinone I treatment dose-dependently induced DNA damage and apoptosis in Huh-7 and HepG2. The network pharmacology analysis and molecular docking showed that EGFR might be potential therapeutic targets of Dihydrotanshinone I in HCC. Our results suggested that Dihydrotanshinone I is a novel candidate therapeutic agent for the treatment of HCC.

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Abstract

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Methods: The proliferation of Huh-7 and HepG2 hepatoma cells was evaluated using the MTT and clone formation assays. An immunofluorescence (IF) experiment of 53BP1 and a flow cytometry analysis were used to detect DNA damage and cell apoptosis, respectively. Moreover, network pharmacology analysis was applied to study the possible therapeutic targets and pathway of Dihydrotanshinone I.

Results: Our results showed that Dihydrotanshinone I effectively inhibited the proliferation of Huh-7 and HepG2 hepatoma cells. Moreover, Dihydrotanshinone I treatment dose-dependently induced DNA damage and apoptosis in Huh-7 and HepG2. The network pharmacology analysis and molecular docking showed that EGFR might be potential therapeutic targets of Dihydrotanshinone I in HCC. Our results suggested that Dihydrotanshinone I is a novel candidate therapeutic agent for the treatment of HCC.

Keywords: Dihydrotanshinone I; hepatocellular carcinoma; proliferation; DNA damage; EGFR

Introduction

Liver cancer is one of the most common causes of death worldwide. Patients are often diagnosed with liver cancer in advanced stages, contributing to its poor prognosis. Of all liver cancer cases, >90% are hepatocellular carcinomas. Classic surgical resection or discharge, chemotherapy often has a variety of defects such as high postoperative recurrence rate, short survival period, large toxic side effects, metastasis, and resistance,

and is greatly limited in actual clinical applications [1]. Therefore, the development of effective anti-cancer drugs has become a research hotspot for HCC treatment.

For centuries, natural products have been a major source of drug development, and many new antitumor drugs, such as paclitaxel and etoposide, are natural products or derived from natural products. Dihydrotanshinone I, a natural product isolated from *Salvia miltiorrhiza Bunge*, has a wide range of pharmacological actions, including antimicrobial, antitumor, and anti-inflammatory effects [2, 3]. Recent studies have shown that Dihydrotanshinone I can inhibit PTEN/AKT/HIF1α, NF-κB and RNA binding protein HU antigen R (HuR) signaling pathway thus blocking the cell cycle and inducing cell apoptosis [4, 5]. However, the role and mechanism of Dihydrotanshinone I in HCC are less reported.

As a comprehensive multidisciplinary concept, network pharmacology, based on system biology and multi pharmacology, provides a new network model of "multi-target, multi-function and complex diseases", which is widely used in the research of natural medical plants [6]. By integrating target prediction and network construction, the potential mechanism of natural medical plants was systematically revealed [7]. In recent years, network pharmacology has also emerged as a powerful tool combining pharmacology which helps to explore the potential targets of natural products. He et al. used a network pharmacology approach to identify potential molecular targets for cannabidiol's anti-inflammatory activity [8]. And via network pharmacology, Seo et al. revealed that triptolide showed a promising inhibitory effects on NF-κB to exert anti-cancer activity. Based on

these, it is promising to explore the potential targets of Dihydrotanshinone I against liver cancer.

Here we aimed to elucidate the effect of Dihydrotanshinone I in HCC and further explore the potential mechanisms. We detected the antiproliferative activities of Dihydrotanshinone I against human hepatocarcinoma cells Huh-7 and HepG2. Besides, the incidence of cellular DNA damage and apoptosis was further examined in each of the cancer cell lines treated with Dihydrotanshinone I. Then by integrating network pharmacology, molecular docking, molecular dynamics simulations, and pharmacological phenotypes, we revealed EGFR and its related signaling pathway as potential targets for therapeutic intervention against HCC.

Materials and methods

Cell culture

Human hepatocellular carcinomas cells, Huh-7 and HePG2 were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were cultured at 37°C under 5% CO_2, concurrently maintained in DMEM/ MEM (Gibco, USA), and supplemented with 10% FBS (Gibco, USA) and 1% penicillin-streptomycin.

Reagent

Dihydrotanshinone I (CAS: 87205-99-0) was purchased from Shanghai Energy Chemical

Company and the reagent was dissolved in dimethyl sulfoxide (DMSO) to appropriate concentrations. Additionally, the same concentration of DMSO was considered as a control group to eliminate error.

Cell viability assay

To test the anti-cancer activity of Dihydrotanshinone I, human hepatocellular carcinomas cells were detected by 3-(4, 5-dimethylthiadiazole-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Huh-7 and HePG2 cells were seeded on a 96-well plate at a density of 8×10^3 per well and treated with or without Dihydrotanshinone I for 48 h. Then the MTT reagent (0.5 mg/mL) was treated for another 4 h. The reaction product formazan was dissolved with 100 μL DMSO and measured the absorbance at 490 nm with a microplate reader (Molecular Devices, USA).

Colony formation assay

Huh-7 and HePG2 cells were cultured in 12-well plates at a density of 1000 cells/well and allow the cells to attach to the wells. The liver cancer cells were cultured with Dihydrotanshinone I with indicated concentrations (0, 2.5, and 5.0 μM) and cultured for around 7 days in standard growth media. The cells were washed three times with cold PBS, fixed with 4% formaldehyde for 15 min, and stained with crystal violet for 5 min at room temperature. The colonies were photographed under a light microscope. Eventually, the crystals were dissolved by 500 μL acetic acid (33%) and calculated by reading

absorbance at 560 nm by using an automated Thermo Fisher Multiskan FC microplate.

Immunofluorescence assays (IF)

Cells on coverslips were fixed with 4% formaldehyde for 15 min, washed with ice-cold PBS three times, and permeabilized in 0.5% Triton X-100 for 30 min at room temperature. Subsequently, the coverslips were blocked with 5% GS (Gibco) for another 1 h and incubated with primary antibody against 53BP1 (cat. no. SAB4503016, Millipore) diluted 1:1600 at 4°C overnight. The next day, the cells were washed by PBST, and incubated with DyLight 488-conjugated anti-Rabbit (cat. no. Sa00013-2, proteintech) diluted 1:100 for 1.5 h at room temperature and mounted with DAPI finally. The fluorescent images were captured by using a Nikon fluorescence microscope.

Cell apoptosis assay

Huh-7 and HePG2 cells were grown in 6-well plates and treated with or without Dihydrotanshinone I (0, 2.5, and 5.0 μM) for 48 h. Liver cancer cells were harvested and washed twice with ice-cold PBS. Subsequently, cells used Annexin V/PI apoptosis assay. The assay was achieved following the protocol provided by the Annexin V/PI apoptosis kit (Sigma) and assessed with a flow cytometer (BD FACSCalibur, BD Biosciences).

Construction of protein-protein interaction (PPI) network

The potential targets of Dihydrotanshinone I was predicted using Pharmapper, while the

116 known liver cancer targets were retrieved using DisGeNET. The common targets among
117 the known cancer targets and Dihydrotanshinone I targets were determined and used to
118 construct a PPI network using STRING. The PPI network was visualized using
119 Cytoscape. The top 10 hub genes in the PPI network were identified using the CytoHubba
120 application and the density of the maximum neighborhood component method.

121 **Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses**

122 Analyses of KEGG pathway enrichment were conducted using the Database for
123 Annotation, Visualization, and Integrated Discovery. All targets in the PPI network were
124 included, and the threshold was set as $p < 0.05$. The plots were generated using the
125 statistical programming language R.

126 **Molecular docking and dynamics simulation**

127 The AutoDock Vina software was used for molecular docking. The crystal structure of
128 EGFR T790M/C797S in complex with EAI045 (5zwj) was retrieved from the Protein Data
129 Bank [9]. AutoDock Tools were used to create all ligands and receptors. The docking box
130 was defined as the center of the original ligands, with a radius of 30-50 Å. The poses of
131 the compounds with the best binding affinity to the targets were generated using PyMOL.
132 Using PyMOL poses of the compound with the highest binding affinity to the targets were
133 created.
134 The molecular dynamics (MD) simulations were performed in the Yinfo Cloud Computing

Platform (YCCP) using AmberTools 20 package [10]. The system was solvated by a truncated octahedron (or cubic) water box using OPC (or TIP3P) water model with a margin of 10 Å. Periodic boundary condition (PBC) was used and the net charge was neutralized by Na+ (or Cl-) ions (or 0.15 M of NaCl). Before the MD simulation, 5000 steps of energy minimization were performed using the steepest descent and conjugate gradient method, respectively. Subsequently, constraints were released and the same 5,000 steps of energy minimization were then run for the entire system. During the MD simulations, the particle mesh Ewald (PME) method was performed to deal with the long-range electrostatic interactions. A non-bonded interaction cutoff of 10Å was employed. Using constraints at a constant volume, the entire system was heated from 0 K to 300 K within 60 ps, and then the solvent density was balanced under a stable system (T = 300 K, P = 1 atm) and sampled for 20 ns.

Western blot assay

Human hepatocellular carcinomas cells Huh-7 and HePG2 were cultured in 6-well plates and treated with Dihydrotanshinone I for 48 h. The cells were lysed with lysis buffer and the protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA). Next, the proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a 10% gel and transferred to a 0.25 μm PVDF membrane. The membrane was blocked in the fresh 5% nonfat milk for 1.5 h at room temperature and incubated with primary antibodies at 4°C overnight (CST, Danvers, USA). The membrane

was washed with PBST and incubated with the peroxidase-conjugated secondary antibodies for another 1.5 h. Finally, the proteins were detected using an enhanced chemiluminescence detection kit (Bio-Rad Laboratories, CA, USA).

Results

Dihydrotanshinone I inhibits the proliferation of HCC cells

The anti-proliferation effect of Dihydrotanshinone I (Fig. 1A) on Huh-7 and HePG2 cells were firstly detected via MTT assay. We found that Dihydrotanshinone I significantly inhibited the proliferation activity of HCC cells in a dose-dependent manner (Fig. 1B, C). More importantly, we found that when we treated with 3 μM Dihydrotanshinone I on liver cancer cells, the cell survival rate of Huh-7 and HePG2 cells was less than 50%, which means Dihydrotanshinone I has excellent anti-liver cancer activity. The colony formation assay results were also consistent with the MTT results. The results exhibited that when we treated with 2.5 and 5.0 μM Dihydrotanshinone I, the colony formation ability was suppressed significantly (Fig. 1D-F).

Please insert Figure 1

Dihydrotanshinone I inhibits cell proliferation by causing DNA damage

DNA damage could lead to inhibition of cancer cell proliferation. To verify whether Dihydrotanshinone I causes inhibition of proliferation by causing DNA damage, we measured DNA damage level by IF assay. The number of 53BP1 foci, which is a marker

for DNA damage response, significantly increased in Dihydrotanshinone I-treated HCC cells with 2.5 and 5.0 μM (Fig. 2A, B). As shown in our results, cyy260 increased the number of 53BP1 foci in a dose-dependent manner, with Dihydrotanshinone I at 5.0 μM reaching ~4 53BP1 foci per nucleus in HCC cells (Fig. 2C, D).

Please insert Figure 2

Dihydrotanshinone I induce cell apoptosis in HCC cells

Excessive DNA damage could lead to apoptosis. To investigate the cell apoptosis level treated with Dihydrotanshinone I, we first stained the cell nuclei with DAPI. We found that the cells treated with 2.5 and 5.0 μM Dihydrotanshinone I induced nuclear shrinkage or fragmentation compared with no significant change in the negative control group (Fig. 3A). Next, the flow cytometry assay was used to further verify the apoptosis of Dihydrotanshinone I-treated cells. We found that when we treated with indicated concentrations of Dihydrotanshinone I, the apoptosis level was significantly increased (Fig. 3B). After statistical analysis, we found that the level of apoptosis was elevated to 30% in Huh-7 cells after 5.0 μM Dihydrotanshinone I treatment and to 40% in HePG2 cells (Fig. 3C, D).

Please insert Figure 3

Identification of potential targets of Dihydrotanshinone I against HCC

To explore the potential targets of Dihydrotanshinone I, we performed a network pharmacological analysis. In detail, 422 liver cancer targets and 218 drug targets were retrieved from DigGeNET and Pharmapper, respectively. By overlapping cancer and drug targets, 35 common targets were found that might be the potential molecular targets of Dihydrotanshinone I (Fig. 4A). Then, protein-protein interactions (PPIs) network was created using Cytoscape (Fig. 4B), which contained 35 nodes and 241 edges with an average number of neighbors of 14.17. In the PPI network, targets such as EGFR, and AKT1, have more liking edges and were highlighted. Furthermore, hub targets were screened from the PPI network using the MCC method, including EGFR, ALB, AKT1, SRC, CASP3, MDM2, MAPK1, MMP9, PPARG, HRAS (Fig. 4C). And according to the results of KEGG analysis, the 35 common targets were enriched in EGFR tyrosine kinase inhibitor resistance, Epithelial cell signaling, endocrine resistance, FoxO signaling pathway, Focal (Fig. 4D). Among these, EGFR tyrosine kinase inhibitor resistance is one of the most significantly enriched KEGG pathways. Given the key role of EGFR in the PPI network and top-ranked in KEGG analysis, we hypothesized that EGFR may involve in the effect of Dihydrotanshinone I on liver cancer cells.

Please insert Figure 4

Dihydrotanshinone I inhibits EGFR downstream signal transduction

Firstly, we performed molecular docking to explore the possible binding mode between Dihydrotanshinone I and EGFR. According to the docking results, Dihydrotanshinone I

showed a -9.4 kcal/mol docking score which was similar with it to the original ligand (EAI045, -9.6 kcal/mol). As shown in Fig. 5A, the parent nuclear structure of Dihydrotanshinone I completely descended deep inside the binding pocket. A strong hydrogen bond was formed between Dihydrotanshinone I and the Lys745 residue of EGFR. Furthermore, according to the 2D presentation of the binding model shown in Fig. 5B, Dihydrotanshinone I exhibited extensive hydrophobic interactions with Ile759, Glu762, Ala763, Leu777, Met766, Leu788. These results suggested that Dihydrotanshinone I may have a prominent binding potential with the EGFR (allosteric binding pockets.). Further molecular dynamics (MD) simulation was adopted to validate the results of the molecular docking. The MD simulation revealed that the root means square distance (RMSD) of the protein backbone of EGFR, was converged after 4ns of simulation and it was stable for the complete simulation run (Fig. 5C). The binding free energy calculations via the MM/PBSA approach showed that electrostatic interaction (ΔEvdw) was a major interacting force between Dihydrotanshinone I and EGFR protein (Table. 1). Overall, these results suggested that Dihydrotanshinone I may target the allosteric binding pockets of EGFR. Since the EGFR involve in the effect of Dihydrotanshinone I on HCC cells according to the results above, we examined the effect of Dihydrotanshinone I on the expression of EGFR. As shown in Fig. 5D and E, the expression of p-EGFR in Huh-7 and HePG2 was suppressed, which means EGFR was the effective target for inhibiting the proliferation of liver cancer cells. Additionally, our

232 results also confirmed that the phosphorylation levels of STAT3 and AKT, the
233 downstream targets of EGFR, were also inhibited (Fig. 5D, E).

234 *Please insert Figure 5 and Table 1*

235

236 **Discussion**

237 HCC is still one of the most common types of malignant cancer and has the second
238 highest rate of cancer-associated mortality in the world [11, 12]. HCC could be treated by
239 chemotherapy besides surgery or transplant clinically [13]. However, the severe side
240 effects, lack of drug targets, and drug resistance of HCC cells have led to the current
241 unsatisfactory treatment of liver cancer, with a 5-year survival rate is around 17% [14,
242 15]. Apoptosis is one of the main reasons for the suppression of tumor cell proliferation
243 for natural products [16]. Particularly, it has been reported that Dihydrotanshinone I
244 exhibited good anti-cancer activity in a variety of malignancies such as ovarian cancer
245 [17] and liver cancer [18] via causing apoptosis of cancer cells, which is consistent with
246 our experimental results. Massive DNA damage could activate the ATM/CHK2 or
247 ATR/CHK1 signaling pathway, which forms a focus that recruits DNA repair proteins
248 around the damage site and thereby inhibits cell proliferation [19]. Our study further
249 confirmed that apoptosis of liver cancer cells was caused by severe DNA damage by
250 treatment with Dihydrotanshinone I.

EGFR as a classical receptor tyrosine kinase is highly expressed at elevated levels in different forms of cancer, which is related to cancer progression and poor prognosis [20]. Therefore, EGFR is still one of the most essential targets for the treatment cancer, including liver cancer. To date, three generations of EGFR tyrosine kinase inhibitors (TKIs) have been developed, bringing huge clinical benefits. However, due to mutations in EGFR such as T790M, L858, and C797S [21], the therapeutic effect of EGFR inhibitors such as gefitinib [22], afatinib [23], and Osimertinib [24] has not been as expected. As a natural product, we demonstrated that Dihydrotanshinone I suppressed the malignant phenotypes of liver cancer cells via regulating EGFR and downstream signaling pathways STAT3 and AKT. Importantly, through molecular docking and MD simulation, we confirmed that Dihydrotanshinone I may target the allosteric binding pockets of EGFR to overcome EGFR resistance mutations. Interestingly, Dihydrotanshinone I has a similar carbonyl group with the known allosteric inhibitor EAI045. We think the structure of Dihydrotanshinone I may be a kind of novel skeleton structure for EGFR allosteric inhibitors, which warrants further research.

Conclusion

This study confirmed that Dihydrotanshinone I exhibited a strong anti-tumor effect on Huh-7 and HePG2 cells. Our network pharmacology analysis and MD analysis results suggested that EGFR is involved in the anti-proliferation activity of Dihydrotanshinone I against liver cancer cells, and Dihydrotanshinone I may target the allosteric binding pockets of EGFR. The in vitro experiments identified that Dihydrotanshinone I could

suppress the expression of EGFR. All the studies revealed that Dihydrotanshinone I is a promoting therapeutic candidate for the treatment of liver cancer.

Acknowledgments

We thank LetPub (www.letpub.com) for its linguistic assistance during the preparation of this manuscript.

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341

Table 1(on next page)

table 1

Table 1 The results of MM/PBSA free energy calculation (kcal/mol).

1

The results of MM/PBSA free energy calculation (kcal/mol).

Energy Component	ΔE_{vdw}	ΔE_{ele}	ΔG_{Tot}
Dihydrotanshinone I	-33.97	-4.74	-25.49

Figure 1

Figure 1

Figure 1 Growth-inhibitory effects of Dihydrotanshinone I against HCC cells lines (Huh-7 and HepG2). (A) The structure of Dihydrotanshinone I. (B, C) Effect of Dihydrotanshinone I on the viability of human HCC cells. Cells were treated with different concentrations of Dihydrotanshinone I for 48 h, and cell viability was measured using the MTT assay. (D, E) Colony formation of Huh-7 and HepG2 cells treated with 2.5 or 5.0 μM Dihydrotanshinone I for around 7 days. Values are the average ± SD of three independent experiments. ***p < 0.001, vs. control group.

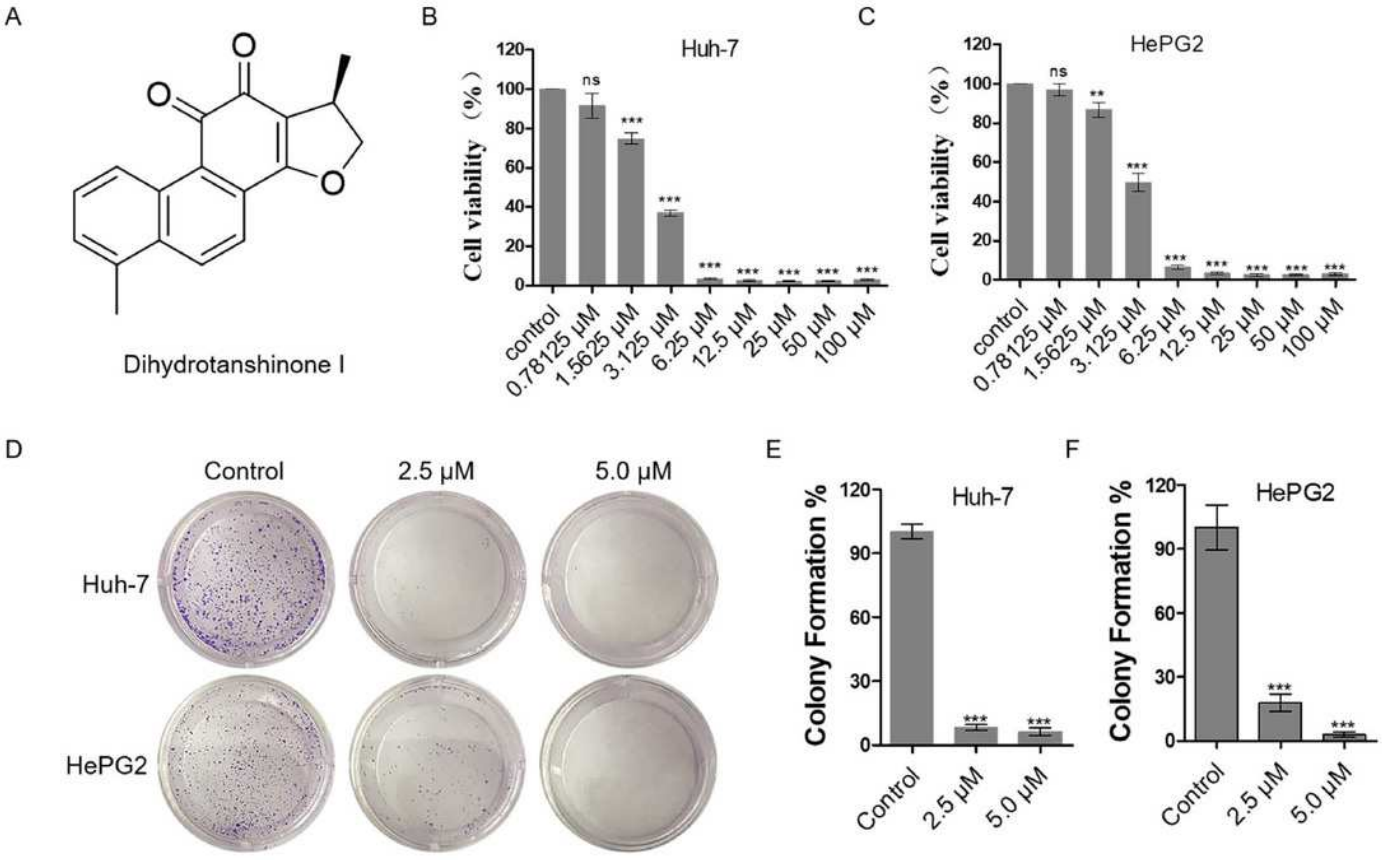

Figure 2

Figure 2

Figure 2. Dihydrotanshinone I treatment provoked strong DNA damage response of HCC cells. (A, B) IF assay for evaluating the DNA damage response. Huh-7 and HepG2 cells were treated with 0.01% DMSO as positive control, 2.5 or 5.0 μM of Dihydrotanshinone I for 48 h before the assay. 53BP1 and DAPI were the DNA damage marker (green) and the nucleus dye (blue), respectively. (C, D) Quantifications of the numbers of 53BP1 foci. 100 cells were counted in each group. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control group.

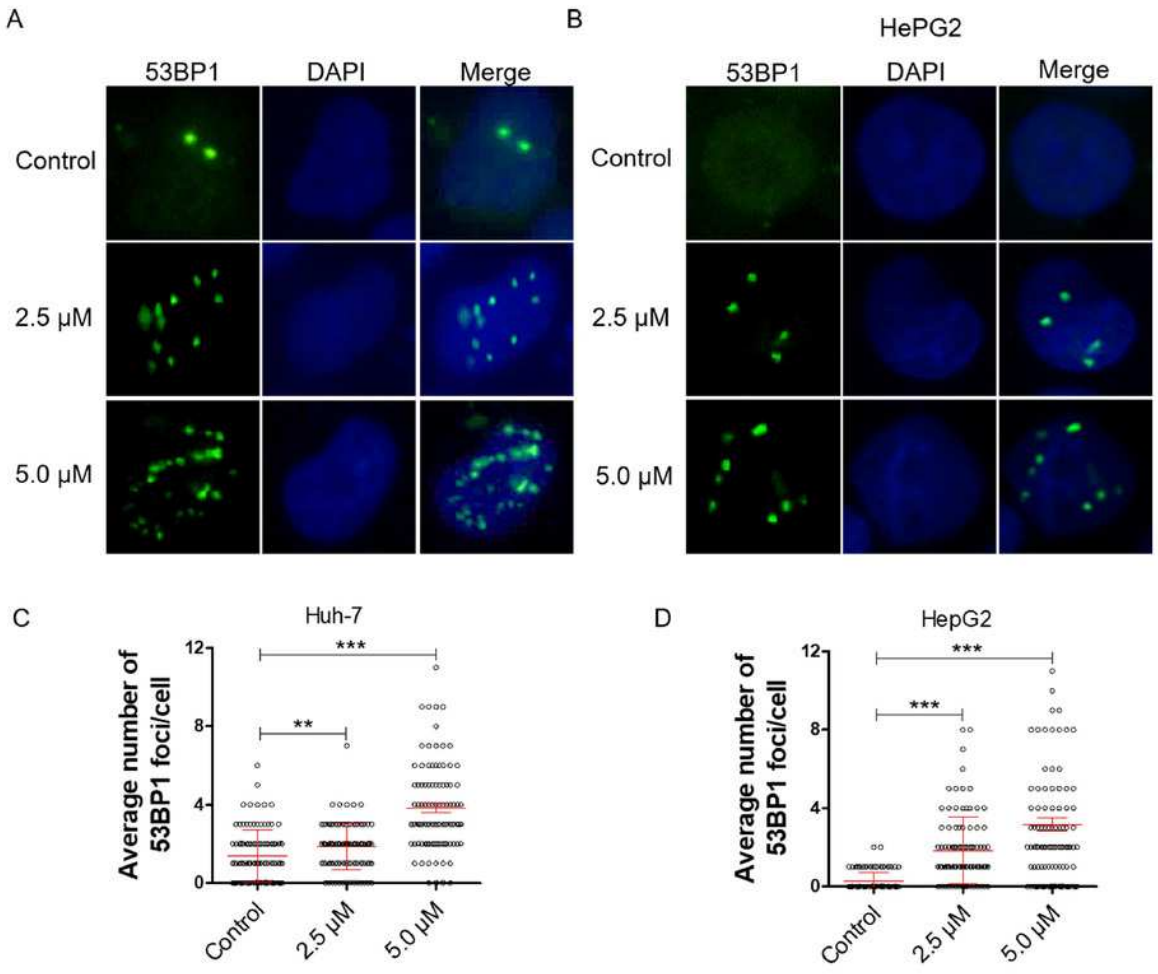

Figure 3

Figure 3

Figure 3. Dihydrotanshinone I induces apoptosis of HCC cells after treated for 48 h. (A) DAPI staining result of HCC cells after treating indicated concentrations of Dihydrotanshinone I for 48 h. (B) HCC cells were treated with 0.01% DMSO as positive control, 2.5 or 5.0 μM of Dihydrotanshinone I for 48 h, HCC apoptosis cells were assayed via FACS analysis. (C, D) Quantification of (B). Values are the average ± SD of three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control group.

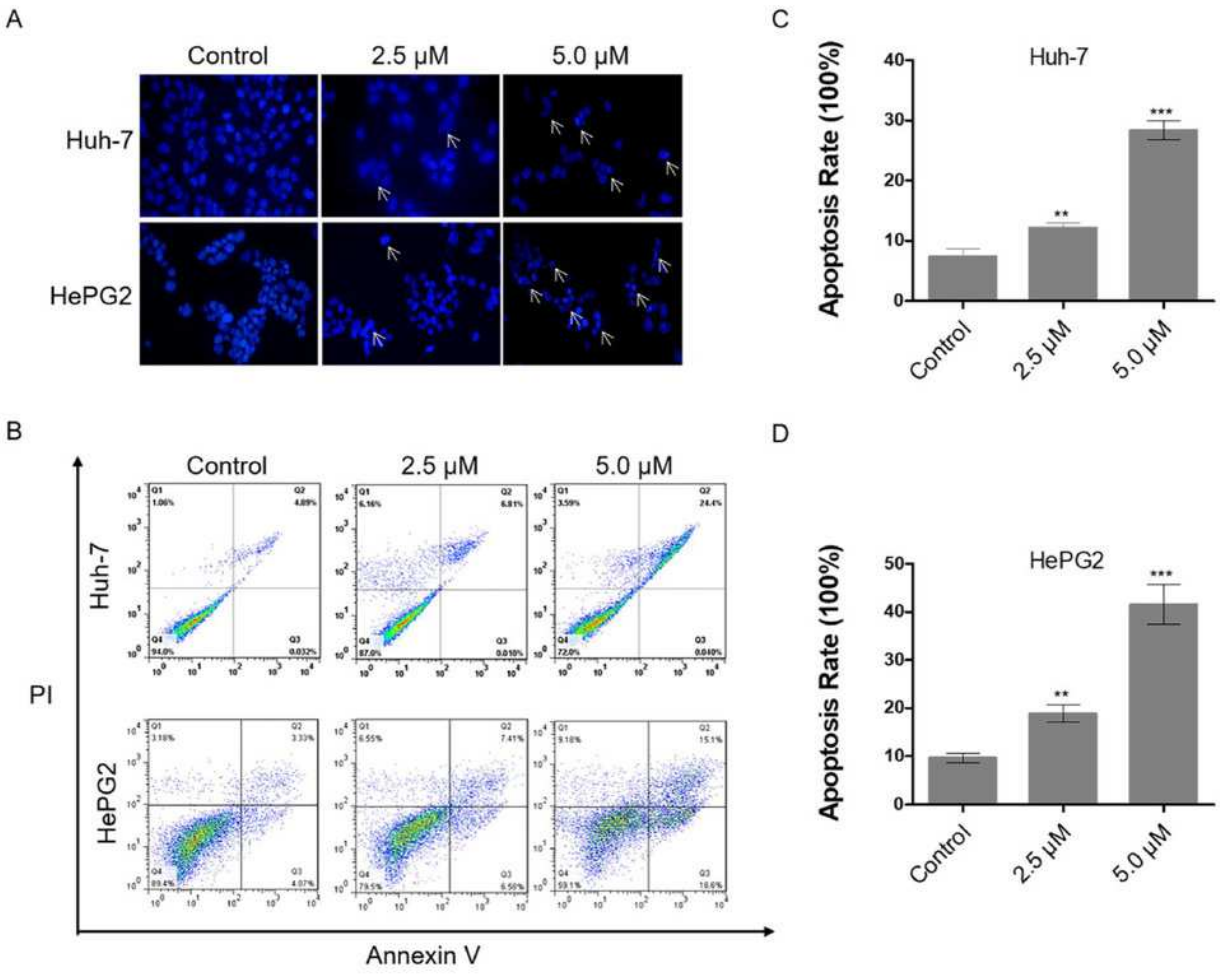

Figure 4

Figure 4

Figure 4. EGFR has a key role in the HCC treatment response. (A) Venn diagram revealing the common targets. (B) Protein-protein interaction network of common targets. (C) Protein-protein interaction network of the top 10 hub genes. (D) KEGG analyze.

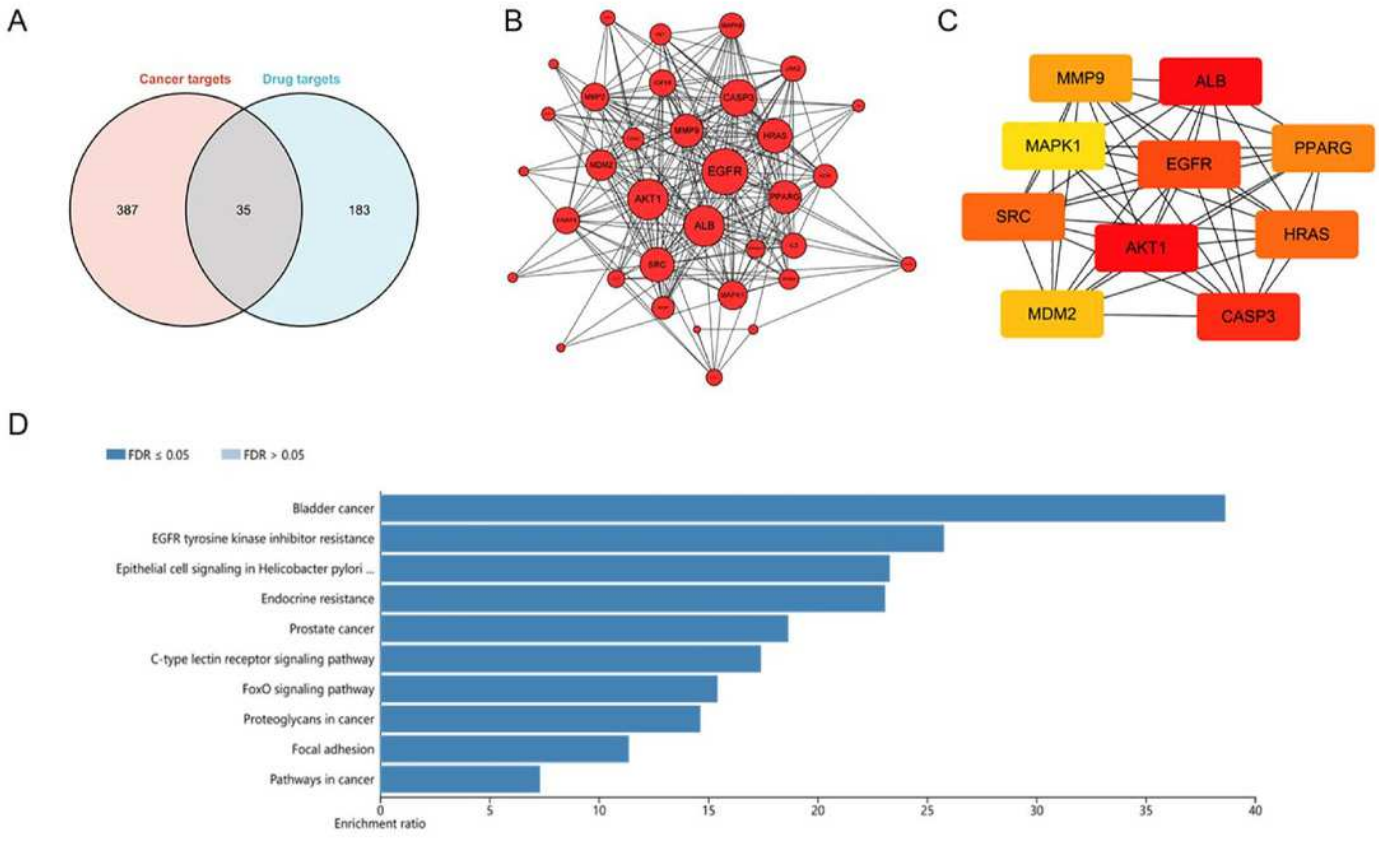

Figure 5

Figure 5

Figure 5. Dihydrotanshinone I suppressed HCC cells proliferation by EGFR and downstream signaling pathway. (A) Calculated binding mode of Dihydrotanshinone I with EGFR. Three-dimensional (3D) presentation of binding mode. (B) Two-dimensional (2D) presentation of hydrophobic interactions between amino acid residues and Dihydrotanshinone I. (C) Simulations of molecular dynamics (MD) with root-mean-square deviations (RMSD). (D, E) Western blot analysis was used to analyze the inhibitory effects of Dihydrotanshinone I on EGFR and its downstream proteins (AKT and STAT3).