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Original Article

Role of ursolic acid chalcone, a synthetic analogue of ursolic acid, in inhibiting the properties of CD133$^+$ sphere-forming cells in liver stem cells

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Abstract: The expression of CD133 decreases with differentiation of tumor cell, indicating that CD133 is a specific marker for isolation and identification of CSCs. In the present study the effect of Ursolic acid chalcone (UAC) on CD133$^+$ hepatocellular carcinoma cell (HCC CSCs) differentiation, their self-renewal, tumorigenic capacity and sensitivity to chemotherapeutic drugs was studied. The results demonstrated that UAC inhibits the expression of CD133$^+$ in a dose and time-dependent manner in PLC/PRF/5 and Huh7 HCC cells. The inhibition was significant at 50 μM and on day 8. The percentage of CD133$^+$ cells decreased from an initial 59.3% in PLC/PRF/5 to 37.1% and 78.2% in Huh7 to 59.2% on treatment with UAC. There was inhibition of Oct4, Tert, Bmi1, *β*-catenin, ABCG2, and tumor sphere-related gene Ep300. In addition it also decreased number of CK19-positive cells and increased number of CK8/18-positive cells. UAC treatment caused a decrease in self-renewal capability and increase in sensitivity to doxorubicin and vincristine drugs in CD133$^+$ HCC CSCs. Therefore, UAC can be a potent therapeutic agent to target differentiation of CSC in HCC.

Keywords: Self-renewal, therapeutic agent, hepatocellular carcinoma, cell differentiation

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer deaths and fifth most common cancer globally [1, 2]. Resistance to chemotherapy and radiotherapy makes overall survival of HCC patients unsatisfactory [3]. The cancer stem cells (CSC) which are rare in tumors exhibit ability of self-renewal, unlimited proliferation, pluripotency and are responsible for tumor recurrence and distant metastasis [4]. CSCs also have stronger resistance to traditional therapies compared to cancer non-stem cells [5-11]. Functional liver cancer stem cells (LCSCs) present in HCC cell lines [12-17] have been isolated using CD133 as a surface maker [14, 18].

Initially CD133 was denoted as primitive hematopoietic and neural stem cell marker [19] but latter on its expression was reported in some normal tissues as well [20-22]. Recently CD133 was detected in CSCs of solid tumors such as brain tumors, renal tumors, colon carcinomas and prostate carcinomas [23-26]. The role of CD133 in maintaining stemness of CSCs is demonstrated by decrease in its expression with differentiation of tumor cells [27]. CD133$^+$ HCC cells are reported to have higher in vitro proliferation capacity and tumorigenesis ability [15].

Ursolic acid, a pentacyclic triterpenoid (**Figure 1**) present in abundance in apple peels [28] suppress tumorigenesis [29], angiogenesis [33] and inhibits tumor promotion [30-32]. Ursolic acid mainly exerts its anti-tumor effect by suppressing the expression of genes regulated by NF-kB [34-40]. Not only ursolic acid but its derivatives also induce apoptosis in a variety of

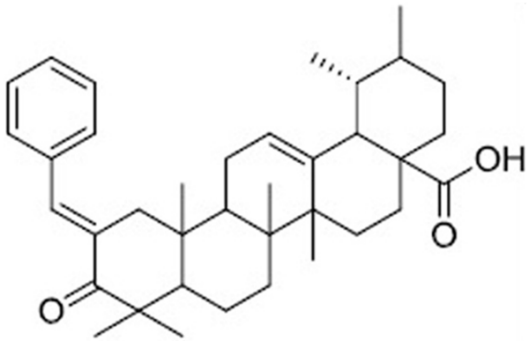

Figure 1. Structure of Ursolic acid chalcone.

cancer cells [41-47] through DNA replication inhibition [48], caspase activation, [43, 45, 47] and tyrosine kinases inhibition [44]. In the present study we first time report the inhibition of CD133+ expression in in HCC CSCs by ursolic acid chalcone.

Materials and methods

Cell culture

The cell lines PLC/PRF/5 and Huh7 were purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China), and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich) supplemented with 10% fetal bovine serum and incubated at 37°C with 5% CO_2. HCC cells were distributed in to 6-well plates (NUNC) in serum free chemically defined medium (CDM) for inducing differentiation. CDM comprised of 1:1 mixture of neurobasal medium and DMEM/F12 medium supplemented with 0.5 × N_2, 0.5 × B27, 0.1% bovine serum albumin, 2 mmol/L glutamine, and 0.1 mmol/L 2-mercaptoethanol, growth factors including UAC (Sigma-Aldrich), 10 ng/mL basic fibroblast growth factor (Millipore), 10 ng/mL EGF (Millipore), 20 ng/mL hepatocyte growth factor (Millipore), 20 ng/mL TGFα (Millipore), and 10 × 7 mol/L dexamethasone (Sigma-Aldrich) were added. For UAC treatment, different amounts of UAC were added to the CDM to reach the indicated final concentration. The agents with no special indication were purchased from the Invitrogen Corporation.

Cell isolation by fluorescence-activated cell sorting or magnetic a ctivated cell sorting

PE-conjugated anti-human CD133/1 antibody (AC133; Miltenyi Biotec) was used to label PLC/PRF/5 and Huh7 cells as per the instructions of manufacturer. CD133+ and CD133- cell subpopulations were sorted by fluorescence-activated cell sorting. The purity of sorted cells was evaluated by flow cytometry, and more than 90% of cells with viability determined by the trypan blue staining were acceptable for the following experiments.

Cell proliferation assay

Three thousand cells were plated in 96-well culture plates for 24 hours and were then treated with different concentrations of UAC for different time durations. Bromodeoxyuridine (BrdUrd) ELISA assay was carried out according to manufacturer's instructions (Roche Diagnostics). ELISA reader (Multiskan MK3; Thermo Scientific) was used to measure Optical density (OD) values at 450 nm.

Statistical analysis

All results were presented as the mean ± SD and analyzed using the Student *t* test. *P* value less than 0.05 was considered statistically significant.

Results

Inhibition of CD133+ expression in HCC by UAC

The results from Western blotting analysis showed a significant decrease in CD133+ expression in PLC/PRF/5 and Huh7, HCC cell lines on treatment with UAC. We observed a decrease in CD133+ expression in dose and time-dependent manner. CD133+ and CD133- PLC/PRF/5 and Huh7 HCC cell were treated with a range of UAC concentrations ranging from 10 μM to 100 μM for 10 days. There was a decrease in CD133+ expression at 30 μM but the effect was significant at 50 μM and maximum at 100 μM concentration of UAC (**Figure 2A**). However, treatment of CD133- cells with UAC could not induce any observable effect. The decrease in CD133 expression in PLC/PRF/5 cells began on day 4 and the expression was minimum on day 8 (**Figure 2B**). Similar results were obtained in Huh7 cells. Thus UAC causes a decrease in CD133 expression in CD133+ PLC/PRF/5 and Huh7 cells at a concentration of 50 μM and exerts maximum inhibitory effect on day 8. Treatment of CD133+ PLC/PRF/5 and Huh7 cells with UAC at a concentration of 50 μM

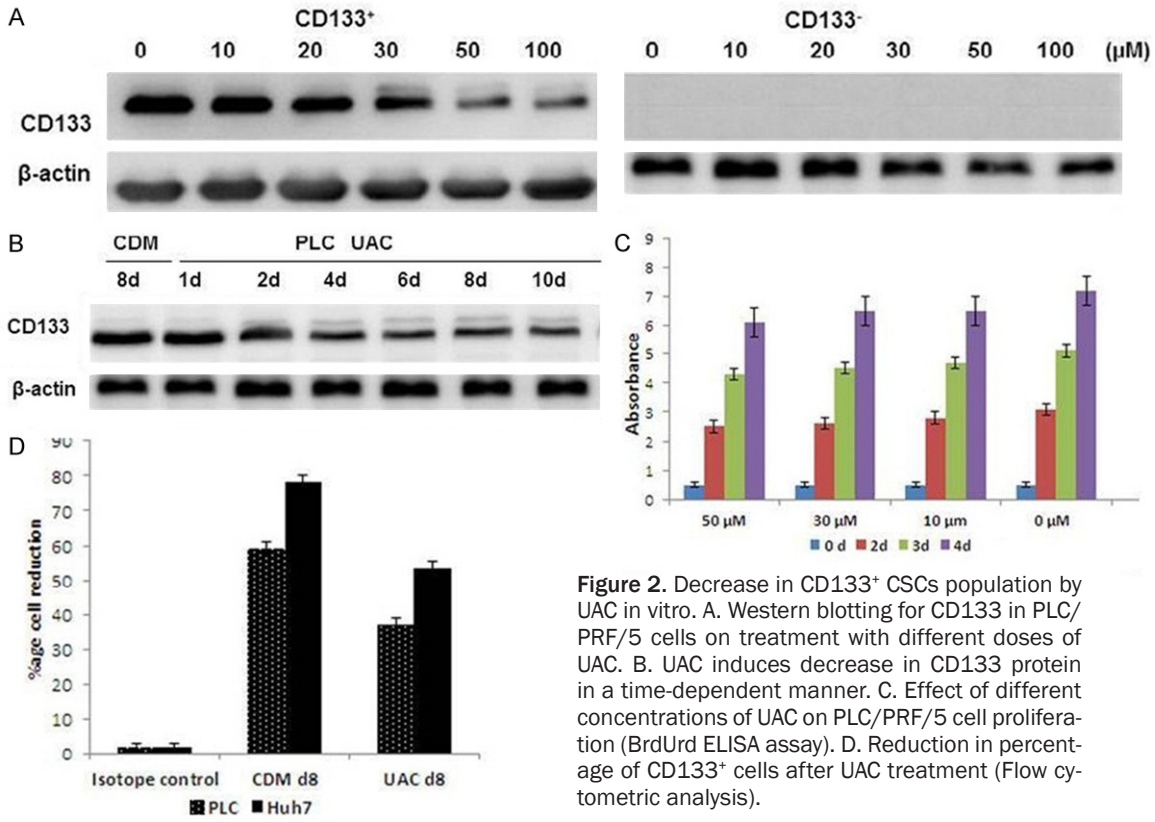

Figure 2. Decrease in CD133+ CSCs population by UAC in vitro. A. Western blotting for CD133 in PLC/PRF/5 cells on treatment with different doses of UAC. B. UAC induces decrease in CD133 protein in a time-dependent manner. C. Effect of different concentrations of UAC on PLC/PRF/5 cell proliferation (BrdUrd ELISA assay). D. Reduction in percentage of CD133+ cells after UAC treatment (Flow cytometric analysis).

resulted in inhibition of cell growth (**Figure 2C**). The percentage of CD133+ cells decreased from an initial 59.3% in PLC/PRF/5 to 37.1%, and 78.2% in Huh7 to 59.2% on treatment with UAC (**Figure 2D**).

UAC induces differentiation of HCC CSCs

Spheroid formation by cancer cells on suspension in CDM enriches CSCs. The self-renewal capability of these cell populations is indicated by the number and size of the spheres [49]. Treatment of CD133+ and CD133- PLC/PRF/5 cell cultures (in suspension with serum-free medium CDM) with UAC resulted in inhibition of stem cell-associated gene expression. The results from Real-time reverse transcriptase PCR analysis showed inhibition of Oct4, Tert, Bmi1, *β*-catenin, ABCG2, and tumor sphere-related gene Ep300 expression in CD133+ cells (**Figure 3A**). However in CD133- cells expression of these genes was not affected on UAC treatment.

Treatment of CD133+ CSCs with UAC led to inhibition of CK19 expression in a time dependent manner (**Figure 3B**). Cytokeratin 19 (CK19) has key role in HCC aggressiveness and acts as a marker for biliary epithelial cells [50]. On the other hand, UAC treatment induced a time-dependent enhancement of CK8/18 expression in the CD133+ CSCs (**Figure 3B**). CK8/18 are hepatocyte-specific markers [51]. However, no effect of UAC treatment was observed on the expression of CK19 and CK8/18 for 8 days in CD133- cells.

Inhibition of self-renewal and tumorigenic capacity of HCC CSCs by UAC

Treatment of CD133+ and CD133- PLC/PRF/5 cell cultures (in CDM suspension) with UAC resulted in lower colony formation efficiency (CFE) in CD133+ PLC/PRF/5 cells on day 8 (**Figure 4A**). However no effect of UAC was observed on CFE in CD133- PLC/PRF/5 cells. The comparison of CFE in CD133+ cells with or without UAC treatment showed that the spheres from former were smaller and only fewer in number than those in latter. Hence UAC treatment caused a decrease in self-renewal capability of CD133+ HCC CSCs.

In an immunodeficient mouse xenograft model, CD133+ cells were injected with UAC or only nor-

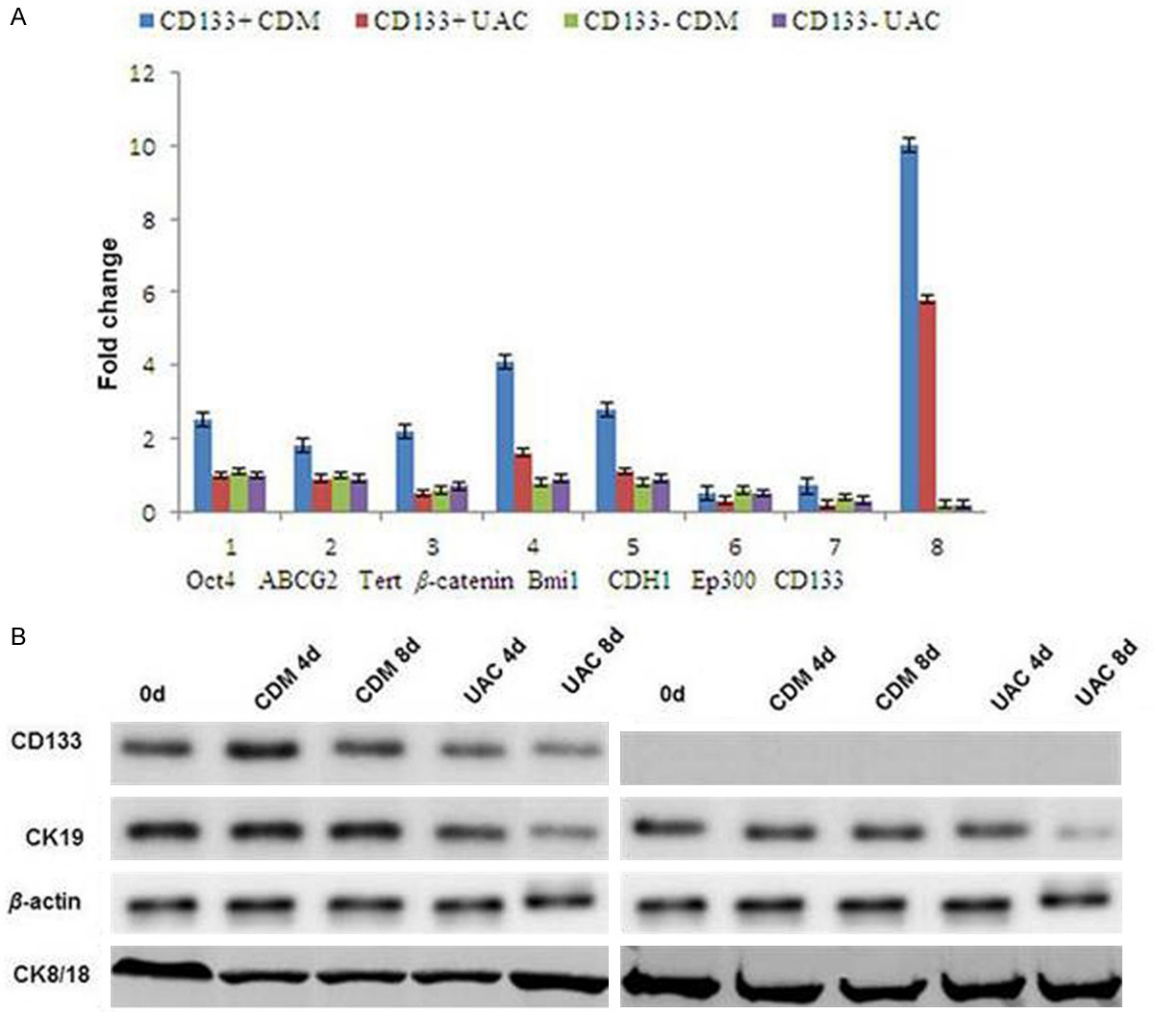

Figure 3. Induction of differentiation in HCC CSCs by UAC. A. The stem-ness-related gene expression of in CD133+ and CD133- cells, cultured as spheres in CDM or in a monolayer with UAC treatment. B. Western blotting shows that UAC enhances CK8/18 expression and decreases CK19 expression in a time dependent manner.

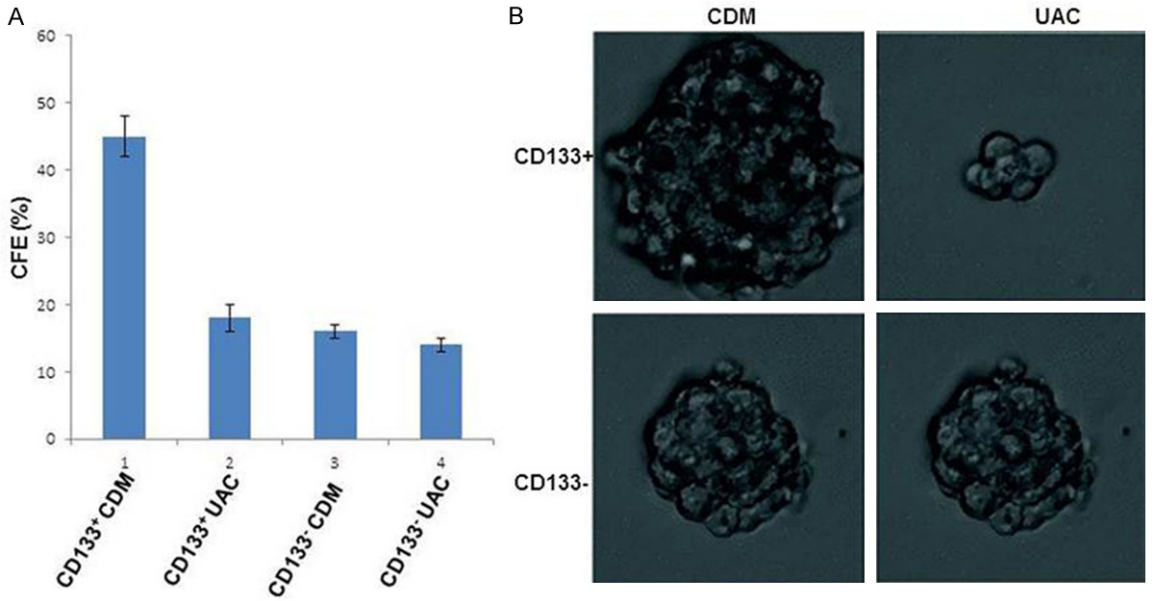

Figure 4. Inhibition of self-renewal and tumorigenic capacities of CD133+ CSCs by UAC. A. CD133+ and CD133- PLC/PRF/5 cells were pretreated with UAC for 10 days. Each group of cells was suspended in growth media containing 0.3% soft agar and seeded in 24-well plates to evaluate colony formation efficiency (CFE; n = 3). B. Inhibition of capacity for CD133+ PLC/PRF/5 cell sphere formation by UAC.

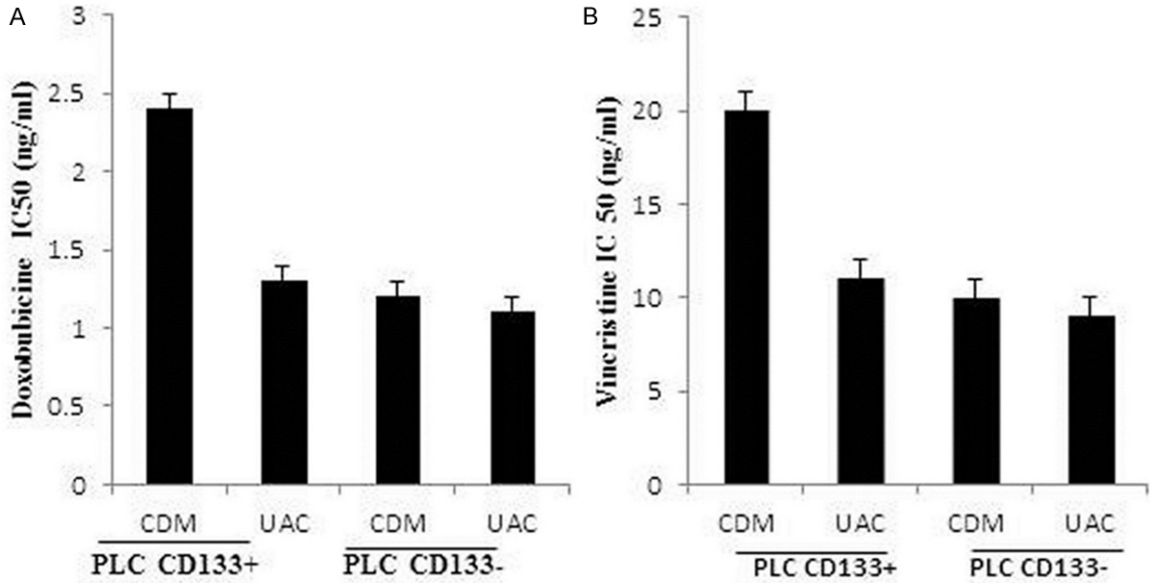

Figure 5. Enhancement in the activity of chemotherapeutic agents on HCC CSCs by UAC. The cytotoxic effects of doxorubicin and vincristine on CD133+ and CD133- PLC/PRF/5 cells that had been pretreated with UAC were tested with the MTT assay.

oped tumors of large size **Figure 4B**, whereas in UAC treated group (10 animals) none of the animals developed tumor. These results demonstrate that UAC exhibits a strong in vivo antitumor effect.

Enhancement of the sensitivity of HCC CSCs to chemotherapeutic drugs by UAC

Resistance to chemotherapeutic agents is a characteristic feature of CSC. Using doxorubicin and vincristine, we demonstrated that UAC treated CD133+ PLC/PRF/5 cells showed increased sensitivity to these drugs. The CD133+ PLC/PRF/5 cells showed marked increase in resistance to doxorubicin and vincristine in absence of UAC (**Figure 5A**). Resistance of HCC CSCs to chemotherapeutic agents is due to upregulation of the superfamily of ABC transporters like ABCG2 [52]. UAC treatment significantly decreased ABCG2 expression in PLC/PRF/5.

Discussion

The role of CD133 in maintaining stemness of CSCs is demonstrated by decrease in its expression with differentiation of tumor cells [27]. CD133+ HCC cells are reported to have higher in vitro proliferation capacity and tumorigenesis ability [15]. Although Ursolic acid and its derivatives have been reported to induce apoptosis in a variety of cancer cells [41-47]. We first time reported inhibition of CD133+ expression by UAC in PLC/PRF/5 and Huh7 HCC cells. Ursolic acid chalcone (UAC) treatment increased CD133+ HCC CSCs differentiation, decreased their self-renewal and tumorigenic capacity and increased their sensitivity to chemotherapeutic drugs. Thus UAC can be a potential differentiation therapeutic agent to target CSCs in HCC. The most successful application of differentiation therapy is the use of all trans retinoic acid in acute premyelocytic leukemia, which is applied as a prodifferentiation inducer to enhance the chemotherapeutic effects [53]. In the present study we observed an increase in CD133+ HCC CSC differentiation on UAC treatment in PLC/PRF/5 and Huh7 HCC cells in dose and time-dependent manner.

The self-renewal capability of HCC CSC cell populations is indicated by the number and size

of the spheres [49]. UAC treatment induced inhibition of Oct4, Tert, Bmi1, β-catenin, ABCG2, and tumor sphere-related gene Ep300 expression. The comparison of CFE in CD133+ cells with or without UAC treatment showed that the spheres from former were smaller and only fewer in number than those in latter. Treatment of CD133+ and CD133- PLC/PRF/5 cell cultures (in CDM suspension) with UAC resulted in lower colony formation efficiency (CFE) in CD133+ PLC/PRF/5 cell after 8 days.

Resistance of HCC CSCs to chemotherapeutic agents is due to upregulation of the superfamily of ABC transporters like ABCG2 [52]. Using doxorubicin and vincristine, we demonstrated that UAC treated CD133+ PLC/PRF/5 cells showed increased sensitivity to these drugs. In conclusion the results from our study demonstrate the ursolic acid chalcone can be a potential candidate in the differentiation theraphy by targeting CSC in HCC.

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Disclosure of conflict of interest

None.

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