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ORIGINAL ARTICLE

Isocyclopamine, a novel synthetic derivative of cyclopamine, reverts doxorubicin resistance in MCF-7/ADR cells by increasing intracellular doxorubicin accumulation and downregulating breast cancer stem-like cells

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Abstract Cyclopamine (CPM) showed promise as a human cancer chemotherapy agent. However, limitations such as stomach acid instability and low solubility impair its clinical application. In this study, we synthesized a novel CPM analogue, isocyclopamine (ICPM), which had comparative bioactivity with CPM and improved stability and solubility. ICPM reversed doxorubicin resistance and had potent synergy with doxorubicin in MCF-7/ADR cells. We further demonstrated that the synergistic mechanism was related to the increased intracellular accumulation of doxorubicin in the cells and the downregulation of the cancer stem-like cells via modulation on both ABCB1 and ABCG2 transporters with independence of Smoothened. The present study identified ICPM as a novel derivative of CPM with better stability and solubility, which provided a useful tool for the biological and medicinal studies, as well as a novel agent for the development of new cancer chemotherapy with improved efficacy.

Keywords Cyclopamine · Isocyclopamine · MCF-7/ADR · ABCB1 · ABCG2

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Introduction

Breast cancer is a common malignant tumor in woman worldwide. Chemotherapy, e.g., doxorubicin (DOX) treatment, is one of the established clinically effective strategies for the treatment of breast cancer. However, the efficacy of DOX is limited by its side effects as well as drug resistance. More and more researches indicate that tumor cell resistance contributes to the failure of chemotherapeutic drugs as treatment [1]. Therefore, novel therapeutic strategies are needed to overcome drug resistance.

One reason that causes the failure of cancer treatment is multidrug resistance (MDR) [2]. MDR reduces intracellular drug accumulation mainly by the active efflux of chemotherapeutic agents through membrane transporters, such as the ATP-binding cassette (ABC) proteins, which include p-glycoprotein (Pgp, MDR1, ABCB1), the multidrug resistance protein 1 (MRP1, ABCC1), and the breast cancer resistance proteins (BCRP, ABCG2) [3]. Another reason for the drug resistance is that the cancer stem-like cells are more resistant to standard chemotherapy drugs [4]. Breast cancer stem-like cells (BCSCs) are a small population of mostly resting cells defined by their long life, high clonogenicity, self-replicating potential, plasticity, highly expression of certain cell surface markers (e.g., aldehyde dehydrogenase (ALDH)), and also drug resistance [5]. Chemical therapeutic strategies targeting the BCSCs may contribute a novel method for overcoming resistance [4]. In recent years, many attempts have been made to attenuate drug resistance, for example, combined chemotherapy using plant-derived compounds and their derivatives [6, 7].

Cyclopamine (CPM, **1**, see Fig. 1), a plant-derived alkaloid product, showed potential effectiveness in tumor therapies in several xenograft models [7, 8]. Combination of low-dose

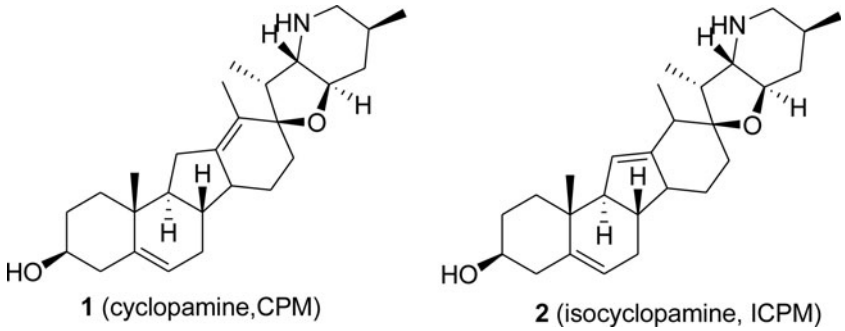

Fig. 1 Structures of cyclopamine (**1**, CPM) and isocyclopamine (**2**, ICPM)

chemotherapeutic agents (e.g., docetaxel, methotrexate, and etoposide) with CPM resulted in enhanced cell killing [9] and antagonized chemoresistance of breast cancer cells both in vivo and in vitro [10], indicating its potent synergy with other chemotherapeutic drugs. Although CPM shows promise as a human chemotherapy agent both used alone or in combination with other chemotherapeutic agents, it has severe inherent limitations that impair its clinical application. Among these limitations are the low stability in stomach acid, low solubility, and moderate biological potency [7, 11, 12]. In this regard, many approaches have been undertaken to increase the potency of CPM and to overcome its pharmacokinetic limitations [11, 13]. In the course of our research on CPM and its derivatives, we synthesized a novel CPM analogue, isocyclopamine (ICPM, **2**, see Fig. 1), which retains the biological profile and potency and has improved stability and solubility. Herein, we described the synthetic methods of ICPM starting from jervine. We also demonstrated that ICPM had the potent synergy with DOX in MCF-7/ADR cells. The mechanism was related to the increased intracellular accumulation of DOX in the cells and the downregulation of cancer stem-like cells.

Materials and methods

Drugs and reagents

Jervine were provided by Nanjing Spring & Autumn Biological Engineering Co., Ltd, China. Antibodies against ABCG2 and ABCB1 were purchased from Santa Cruz Biotechnology, Inc. Antibodies against ALDH, γ-H2AX, cleaved PARP, and procaspase-3 were purchased from Cell Signaling Technology. Phycoerythrin-conjugated anti-human CD24 antibody and FITC-conjugated anti-human CD44 antibody were the products of Invitrogen. Other reagents and kits were the product of Beyond, China.

Cell lines and cell culture

The human lung adenocarcinoma A549, H1975, human colorectal carcinoma HCT-116, human cervical carcinoma HeLa were obtained from the American Type Culture Collection. Human myelogenous leukemia K562, HL-60 cell lines, MCF-7, and MCF-7/ADR cells were provided by the Cell Bank of Chinese Academy of Sciences. A549 cells were cultured in F-12K medium with 10 % fetal bovine serum (FBS). HeLa cells were cultured in Dulbecco's modified Eagle's medium with 10 % FBS. The others were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium with 10 % FBS.

Syntheses of ICPM and acid stability assay

To a mixture of 50 g jervine in 2.5 L diethylene glycol, PEG400 (50 mL), 85 % $NH_2NH_2{\cdot}H_2O$ (150 mL), and KOH (50 g) were added. The reaction mixture was stirred at 160 °C for 2 h. The reaction mixture was concentrated in vacuum to remove the water. Then, the reaction mixture was stirred at 180 °C for 2 h. The reaction mixture was added water with stir and filtered to collect the solid. The residue was purified by pre-HPLC, and the pure compounds were identified by ^1H and ^{13}C NMR. Acid stability assay was performed by subjecting the compound to a mixture of $D_2O/DCl/CD_3OD$ with the pH about 1.5, and ^1H NMR spectra were recorded at different time periods.

Cell viability assessment

The cytotoxicity of CPM and ICPM alone in combination with other chemical drugs was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cancer cells were untreated or treated with certain concentrations of compounds for 72 h. MTT (20 μL, 0.5 mg/mL) was added and incubated for another 4 h, and then, the supernatant was removed, and the dye crystals were dissolved in 200 μL DMSO. Absorbance was measured at 490 nm using a microplate reader (BioTek, USA).

Drug combination and calculation of synergism

MCF-7/ADR cells were seeded in triplicate in 96-well plates and treated with DOX alone, ICPM alone, or with the combination of these two drugs, at the indicated doses. MTT assays

were performed after 72 h of treatment. The medium-effect method was employed to analyze concentration-response data for single drug or multiple drugs. The synergistic effect of multiple drugs was calculated by the definition of Chou and Talalay [14]. The Chou and Talalay combination index (CI) reflecting the interaction of two drugs was calculated at different levels of growth inhibition with the use of software package Calcusyn (Biosoft, Cambridge, UK). The CI values of <1, 1, and >1 indicate synergistic, additive, and antagonistic effects, respectively.

Intracellular DOX accumulation and efflux assay

Flow cytometry was used to quantify the intracellular accumulation and retention of DOX. A total of 2×10^5 cells/well were seeded on 6-well plates. After overnight incubation, cells were incubated with 1.5 μM DOX in the absence and presence of different concentration of ICPM for 24 h. In relation to the intracellular retention of DOX, MCF-7/ADR cells were incubated for 12 h with 5 μM DOX followed by incubation for another 6 h in normal culture medium or in medium containing certain concentration of ICPM. Cells were harvested after trypsin treatment and washed twice. Then, the autofluorescence of DOX was measured by flow cytometer moflo XDP (Beckman-Coulter, Fullerton, CA), using the excitation with an argon laser at 488 nm and detected on FL2 channel.

SP analysis

Side population (SP) analysis in MCF-7/ADR cell lines was performed using Hoechst 33342 flow cytometry method. In brief, MCF-7/ADR cells were plated onto 6-well plates and treated with ICPM (0–50 μM) and CPM (25 μM) for 48 h. Cells were trypsinized and washed with phosphate-buffered saline (PBS). Cells (1×10^6 cell/mL) were then suspended in RPMI media supplemented with 2 % FBS and 1 mM HEPES buffer with or without verapamil (50 μM) for 10 min. Hoechst 33342 was then added followed to a final concentration of 2.5 μg/mL and incubated for 90 min at 37 °C. Cells were recovered by centrifugation and washed several times with ice-cold PBS to remove unbound dye and finally suspended in ice-cold PBS containing 2 % FBS. PI (2 μg/mL) was added, and SP cells were analyzed by flow cytometer moflo XDP (Beckman-Coulter).

Western blotting assay

After treated with certain concentrations of different drugs, MCF-7/ADR cells were collected, washed, and lysed with RIPA buffer containing fresh protease inhibitor mixture. Protein samples were resolved on 10–15 % SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with protein-specific antibodies followed by HRP-conjugated secondary antibody. Bands corresponding to the antibodies were detected by FluorChem E (ProteinSimple, USA).

Colony formation assay

MCF-7/ADR cells were plated onto 6-well plates, at a density of 500 cells per well; ICPM (0–12.5 μM) and CPM (12.5 μM) was added to medium 48 h after seeding when indicated. After incubation for additional 16 days, cells were washed, fixed in methanol for 15 min, and stained with Giemsa for 30 min. Colonies consisting of >50 cells were scored.

Cell migration assay

MCF-7/ADR cells were counted and plated in 24-well plates. After growth to 90 % confluence, "scratch" wounds were created by scraping cell monolayers with sterile disposable yellow tips. After washing the scratched cell monolayers with PBS twice, RPMI 1640 medium supplemented with 2 % FBS containing certain concentrations of ICPM was added. After incubation for 48 h, three fields of each wound were selected and photographed with an inverted phase contrast microscope. The width of the remaining wound was measured, and the migration inhibition rate was calculated using the following formula:

$$\text{Inhibition rate}\,(\%) = [1 - (\text{average width in drug treated group}/\text{average width in control group})] \times 100\,\%$$

Mammosphere formation assay

The mammosphere forming assay was performed as described previously with slight modification [15]. Briefly, the MCF-7 cells were plated in ultra-low-attachment 6-well plates (Corning, Acton, MA, USA) at a density of 2000 cells/well in primary culture, which were supplemented with 2 mM L-glutamine, 2 % B27 supplement, 10 ng/mL human recombinant epidermal growth factor and 20 ng/mL basic fibroblast growth factor, 5 μg/mL insulin, and the tested compounds ICPM (0–6.25 μM) and CPM (6.25 μM). Mammospheres were counted

after culture for 21 days under microscope, and photographs were acquired.

CD44 and CD24 staining

The MCF-7/ADR cells were plated in 6-well plates and cultured for 24 h. After treatment of ICPM for 48 h, the MCF-7/ADR cells were stained with phycoerythrin-conjugated anti-human CD24 antibody and FITC-conjugated anti-human CD44 antibody according to the manufacturer's instructions. Samples were analyzed using a flow cytometer moflo XDP (Beckman-Coulter).

Data analysis

Student's *t* test was performed for all statistical analysis, and values were expressed as mean ± SD. $P<0.05$ was considered significant.

Results

Preparation and acid stability of ICPM

After the reduction of jervine (see Fig. 2a), the products were purified by pre-HPLC to give two pure compounds, **1** (70 %) and **2** (30 %). Product **1** was identified as cyclopamine. Product **2**, termed as isocyclopamine, was identified as a new compound by the ^{1}H, ^{13}C, HSQC, and HMBC NMR spectra. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.40 (d, 1H, H-11), 5.37 (m, 1H, H-6), 3.53 (tt, J=11.2, 4.0 Hz, 1H, H-3), 3.21 (ddd, J=10.8, 9.7, 3.9 Hz, 1H, H-23), 3.03 (ddd, J=12.4, 4.2, 1.1 Hz, 1H, H-26), 2.51 (t, J=8.8 Hz, 1H, H-22), 2.41 (p, J=7.6 Hz, 1H, H-20); 2.37 (ddd, J=10.8, 4.8, 2.2 Hz, 1H, H-4), 2.23 (m, 1H, H-4), 2.18 (m, 1H, H-11), 2.11 (m, 1H, H-24), 2.0 (m, 1H, H-14), 2.0 (m, 1H, H-9), 1.89 (m, 1H, H-16), 1.83 (m, 1H, H-2), 1.81 (m, 1H, H-15), 1.78 (m, 1H, H-7), 1.76 (m, 1H, H-1), 1.58 (m, 1H, H-25), 1.55 (m, 1H, H-2), 1.51 (m, 1H, H-16), 1.31 (m, 1H, H-8), 1.25 (m, 1H, H-15), 1.23 (m, 1H, H-1), 1.13 (m, 1H, H-24), 1.10 (s, 3H, H-18), 0.98 (d, J= 5.6 Hz, 3H, H-21), 0.94 (d, J=5.2 Hz, 3H, H-27), 0.92 (s, 3H, H-19); ^{13}C NMR (100 MHz, $CDCl_3$) δ 150.1 (C-12), 141.8 (C-5), 122.6 (C-11), 121.4 (C-6), 86 (C-17), 76.2 (C-23), 71.9 (C-3), 64.8 (C-22), 59.6 (C-9), 54.5 (C-26), 48.7 (C-14), 45.2 (C-13), 42.5 (C-4), 41.9 (C-7), 40.3 (C-10), 38.9 (C-20), 38.3 (C-1), 37.1 (C-8), 31.5 (C-25), 31.4 (C-2), 31.4 (C-24), 30 (C-16), 27.5 (C-15), 19.1 (C-27), 18.9 (C-19), 10.9 (C-18), 10.5 (C-21).

^{1}H NMR was used to evaluate the stability of ICPM. ICPM was subjected to a mixture of D_2O/DCl/CD_3OD at pH 1.5. While CPM would decompose at such an acidic condition [11], ICPM showed no signs of decomposition according to its ^{1}H NMR spectra even after 24 h of acid treatment (Fig. 2b). These results suggested that ICPM was stable under acidic condition. During the NMR sample preparation, we noted that the solubility of ICPM in methanol was more than 12 mg/mL, which was twofold more soluble than CPM (5 mg/mL in methanol). However, as CPM was, ICPM was not soluble in water.

ICPM potentiates anti-cancer effect of DOX in MCF-7/ADR cells

To evaluate the cytotoxicity of ICPM in vitro, we examined its proliferative inhibition on several human tumor cell lines. As shown in Fig. 3a, ICPM displayed moderate cytotoxicity on the tested human cancer cell lines with the IC_{50} values ranging from 23 to 45 μM, most of which were slightly lower than that of CPM (37–69 μM). In the case of MCF-7/ADR cell line which is resistant to DOX, ICPM inhibited the proliferation (IC_{50} is about 41 μM), while CPM did not, even up to 100 μM. The results indicated that the cytotoxicity of ICPM in vitro was moderate, and a little more potent than CPM. In addition, we observed obvious precipitation of CPM in the cell culture medium at a concentration of 50 μM, but no precipitation of ICPM, which further confirmed the better solubility of ICPM in cell systems.

Considering that CPM has been reported to reverse multiple drug resistance, we evaluated whether ICPM could reverse the DOX resistance in MCF-7/ADR cells, as well as the potential synergistic effect of ICPM with DOX. Our results showed that DOX or ICPM alone had no obvious inhibitory effect on the cell density, whereas addition of ICPM significantly increased DOX-induced growth inhibition in the resistant cells (Fig. 3b); the IC_{50} values of DOX in MCF-7/ADR cells decreased from 52.9 to 22.1, 10.54, and 3.43 μM, in the presence of ICPM of 2.5, 5, and 10 μM, respectively (Fig. 3c). The median effect analysis showed that the CI was smaller than 1 (Fig. 3d), further indicating the potent synergism between ICPM and DOX on the inhibition of cell proliferation. ICPM also markedly enhanced DOX-induced apoptosis in MCF-7/ADR cells comparing to ICPM or DOX administrated alone, as indicated by the increased level of apoptotic markers, including cleaved PARP and γ-H2AX, as well as the decreased level of anti-apoptosis proteins such as procaspase 3 (Fig. 3e), suggesting that ICPM could reverse the DOX resistance in MCF-7/ADR cells. These data demonstrated that ICPM had a potent synergism with DOX in MCF-7/ADR cell lines, although its direct cytotoxicity in vitro was relatively weak.

ICPM increases the intracellular accumulation and decreases the efflux of DOX in MCF-7/ADR cells

In order to evaluate the intracellular accumulation of DOX, MCF-7/ADR cells were first incubated for 24 h with 1.5 μM

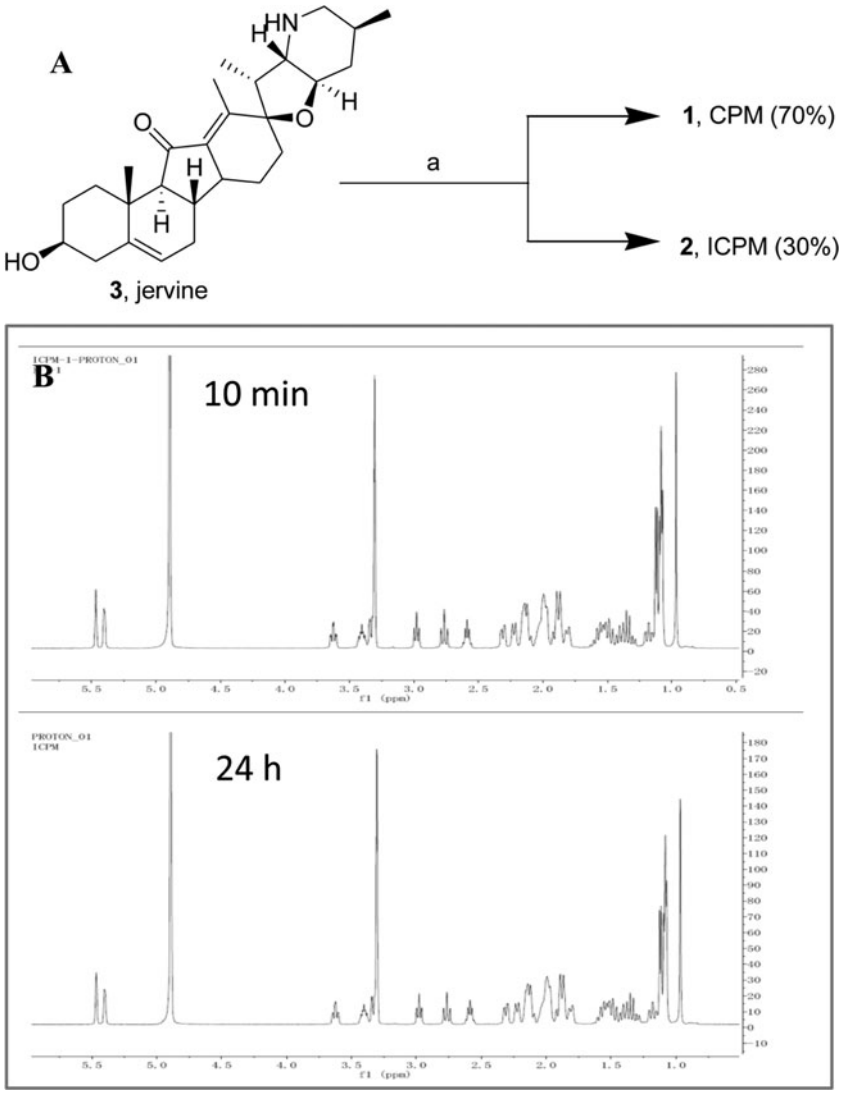

Fig. 2 Synthesis scheme of isocyclopamine and its acid stability. **a** Synthesis of CPM (**1**) and ICPM (**2**) from jervine (**3**). *a* Diethylene glycol, PEG400, $NH_2NH_2{\cdot}H_2O$, KOH, 160 °C, 2 h. **b** ^1H NMR spectra of ICPM in $D_2O/DCl/CD_3OD$ at pH 1.5 at 10 min (*upper panel*) and 24 h (*lower panel*)

DOX alone or in combination with 12.5 to 50 μM ICPM or 25 μM CPM; the autofluorescence of DOX was then measured by flow cytometry. ICPM itself gave a negligible fluorescence and did not influence the DOX signal (data not shown). Only a small amount of DOX accumulated in cells after treatment of DOX for 24 h. In contrast, cells treated with DOX in combination with ICPM or CPM accumulated much more DOX (Fig. 4a). The increased accumulation of DOX induced by ICPM was in a concentration-dependent manner. Similar effects were observed for ICPM and CPM at the concentration of 25 μM (Fig. 4b). These findings indicated that both ICPM and CPM increased the intracellular accumulation of DOX.

We further evaluated the intracellular retention of DOX affected by ICPM. MCF-7/ADR cells were incubated for 12 h with 5 μM DOX followed by incubation for another 6 h in the absence or presence of certain concentration of ICPM. As shown in Fig. 4c, only a small amount of previously accumulated DOX remained in cells after withdrawal of DOX for 6 h. However, when treated with ICPM or CPM, cells contained much more DOX than those incubated in normal growth medium after DOX withdrawal. These results indicated that ICPM also increased the intracellular retention of DOX. At 25 μM ICPM, the result starts showing increased intracellular retention of DOX; much significant effect was observed at 50 μM ICPM (Fig. 4d). CPM and ICPM at the concentration of 25 μM had a similar increase in the retention of DOX (Fig. 4d). All of these findings showed that ICPM increased both the intracellular accumulation and retention of DOX in MCF-7/ADR cells.

ICPM decreases the SP cell fraction in MCF-7/ADR cells

SP cells, which contain a relative high percentage of cancer stem cells, contributed to the drug resistance properties of many cancer cells, including the breast cancers [16]. To investigate whether ICPM has an effect on the SP cells of MCF-7/ADR cells, we examined the change of the SP percentage after

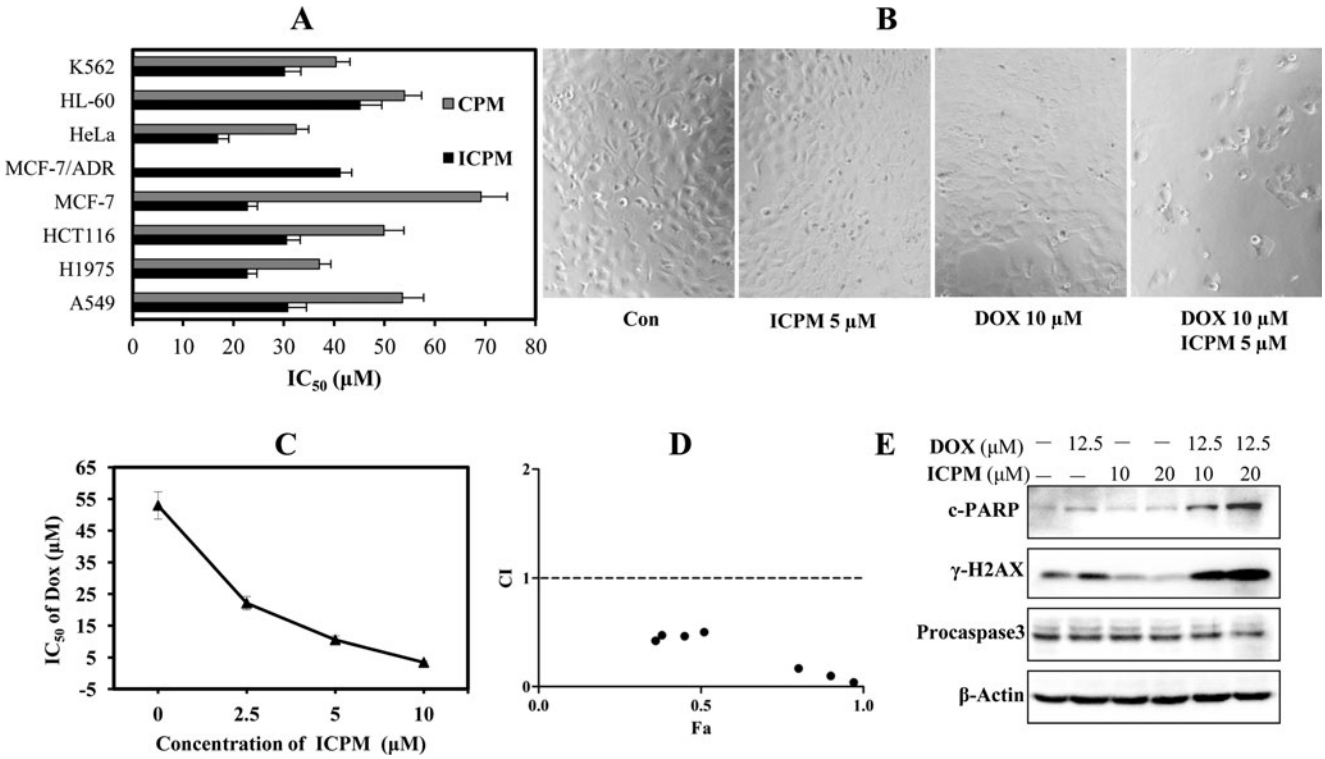

Fig. 3 ICPM reverses the resistance of DOX in MCF-7/ADR cells. **a** ICPM displays moderate cytotoxicity on the tested human cancer cell lines assayed by the MTT methods, after treatment with ICPM for 72 h. **b** Representative pictures showing the effect on cell density of DOX alone (10 μM), ICPM alone (5 μM), or in combination for 72 h. **c** ICPM decreases the IC_{50} values of DOX against MCF-7/ADR cells. Cells were treated with DOX for 72 h in the presence of 2.5, 5, and 10 μM ICPM, and the IC_{50} values of DOX were calculated, respectively. Growth inhibition was evaluated by MTT assay. **d** ICPM shows synergistic effect with DOX. Cells were treated with DOX (12.5 μM) in the presence of 1.25 to 80 μM ICPM for 72 h. The combination index (CI) was calculated by Calcusyn software. CI smaller than one indicates synergism between ICPM and DOX. **e** ICPM enhances DOX-induced apoptosis in MCF-7/ADR cells. Cells were treated with ICPM (10, 20 μM), DOX (12.5 μM) alone, or in combination for 24 h, and the apoptosis-related proteins were detected by Western blotting as described in the "Materials and methods" section

treatment with different concentrations of compounds. The percentage of SP cells decreased from 85.3 % in the control group to 0.35 % of the total MCF-7/ADR cells after preincubating with verapamil (50 μM) for 10 min (Fig. 5a), which validated our experimental conditions for the placement of SP gate. The percentage of SP cells in MCF-7/ADR cells was reduced from 86.6 % in vehicle control cells to 74.2, 72.1, and 57.6 % after treatment with 12.5, 25, and 50 μM ICPM, respectively. Similarly, the percentage of SP cells in MCF-7/ADR cells was reduced to 79.3 %, after treatment with 25 μM CPM (Fig. 5b). Our data showed that ICPM significantly inhibited SP in a dose-dependent manner.

ICPM reverses the drug resistance in MCF-7/ADR via ABCB1 and ABCG2

ABCG2 and ABCB1-mediated MDR can be reversed either by inhibiting their transport activity or by decreasing their protein levels. To further explore the effect of ICPM on the expression levels of ABCG2 and ABCB1, Western blotting was performed after incubation with the compounds for 48 h. As shown in Fig. 6a, the results showed significant decreases in the protein levels of both ABCB1 and ABCG2 in MCF-7/ADR cells, in a concentration-dependent manner. ICPM had comparable ability to decrease both ABCB1 and ABCG2 protein levels, when compared with CPM (Fig. 6b). In addition, in the presence of DOX, which is required to maintain the drug resistance prosperity in MCF-7/ADR cells [17], ICPM still decreased the expression level of ABCB1 and ABCG2, with a much more significant effect on ABCG2 (Fig. 6c, d).

To further confirm the modulation on ABCB1 and ABCG2 by ICPM, different chemotherapy substrates of ABCB1 and ABCG2 were employed and their sensitivity in MCF-7/ADR cells was assayed in the absence and presence of ICPM. In DOX-resistant MCF-7/ADR cells highly expressing ABCG2 and ABCB1, ICPM also sensitized other chemotherapeutic agents' cytotoxicity including colchicine (COL) and 5-fluorouracil (5-FU), which are the substrates of ABCB1 and ABCG2 [18], respectively (Fig. 6e, f). In contrast, as shown in Fig. 6g, in MCF-7/ADR cells, ICPM could not enhance the sensitivity of cisplatin, which is neither the substrate of ABCG2 nor ABCB1 [15]. Furthermore, no enhancement of DOX sensitivity in the presence of ICPM had been detected in MCF-7 cell line (Fig. 6h), which expresses low level of

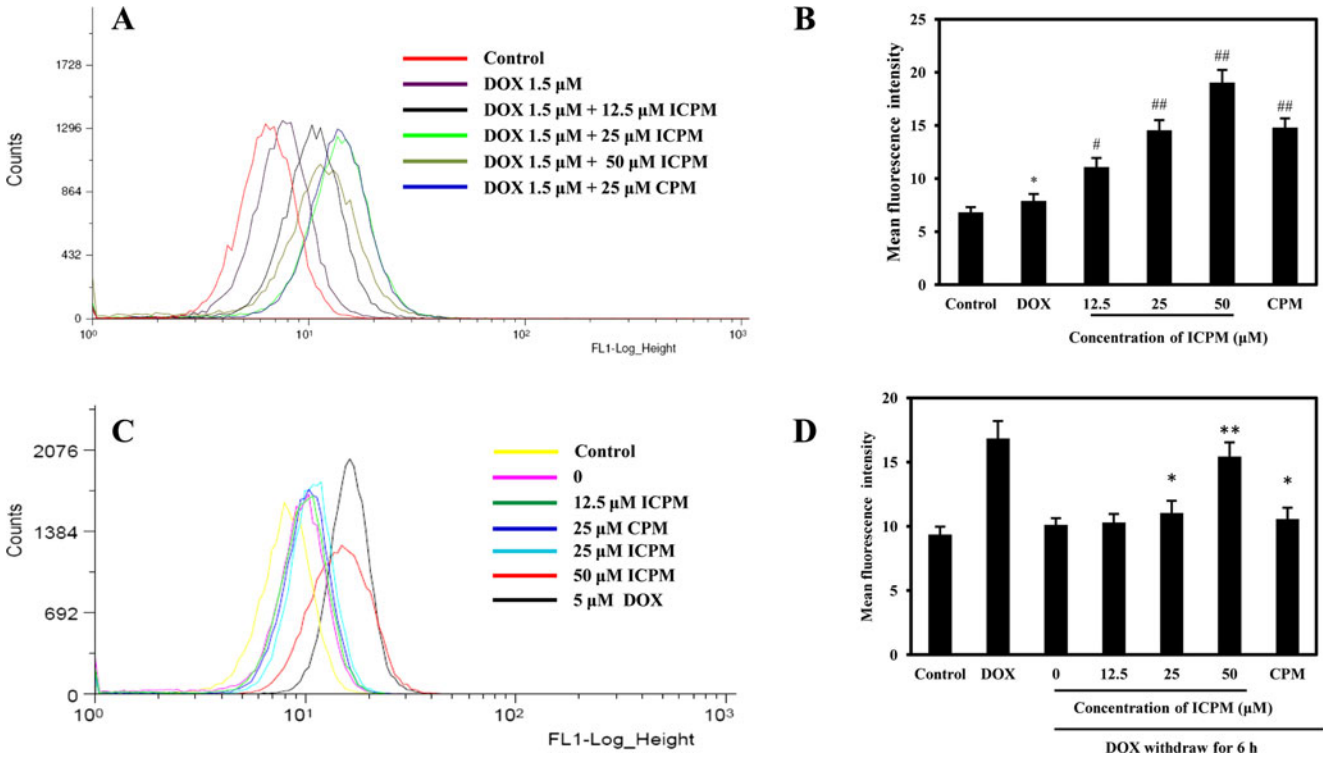

Fig. 4 ICPM increases the intracellular accumulation and retention of DOX in MCF-7/ADR cells. **a** Fluorescent intensity of MCF-7/ADR cells that were incubated for 24 h in medium containing DOX alone (1.5 μM) or in combination with ICPM (12.5–50 μM) or CPM (25 μM). **b** Histograms of the fluorescence intensity of DOX detected by flow cytometer moflo XDP (Beckman-Coulter). Values represent the means ± SD of triplicate measurements. *$P<0.05$, **$P<0.01$ versus medium control. $^{\#}P<0.05$, $^{\#\#}P<0.01$ versus DOX group. **c** Fluorescence intensity of DOX in MCF-7/ADR cells incubated with DOX (5 μM) for 12 h followed by further 6 h incubation in normal growth medium or medium containing various concentrations of ICPM or CPM. **d** Histograms of the mean fluorescence intensity of intracellular DOX. Values represent the means ± SD of triplicate measurements. *$P<0.05$, **$P<0.01$ versus medium control

ABCG2 and ABCB1. The results further confirmed that ABCG2 and ABCB1 were involved in the recovery of DOX resistance and ICPM inhibited the transporter function of both ABCG2 and ABCB1.

ICPM suppresses clonogenic potential and metastatic capacity of MCF-7/ADR cells in vitro

As ABCG2 is not only a drug efflux pump but also a modulator within CSCs [19], we then evaluated whether ICPM-induced ABCG2 dysfunction could affect the CSC properties of MCF-7/ADR cells. CSC properties usually show a high proliferative, clonogenic, and metastatic capacity [5]. To further determine whether ICPM decreases the CSC properties of MCF-7/ADR, we first tested its effect on colony-forming ability. ICPM decreased the colony production significantly compared to the control group (Fig. 7a), and the inhibition was in a concentration-dependent manner (Fig. 7b). At 12.5 μM, ICPM had similar inhibition ability as did CPM, which all abolishing the formation of colonies completely (data not shown). These results indicated that ICPM possessed a high function to inhibit the clonogenic potential of MCF-7/ADR. In addition, as migration is one of the characters for CSC properties, we investigated the effect of ICPM on MCF-7/ADR cell motility using scratch-wound assay. The results from the scratch-wound assay showed that the wound healing was gradually reduced as ICPM concentration increased (Fig. 7c), and the inhibition rate was in a concentration-dependent relationship (Fig. 7d). Although these results are suggestive of ICPM suppressing clonogenic and metastatic capacity, which is associated with CSC properties, it should be noted that not only CSCs possess these properties, but some normal cancer cells also do. Therefore, more specific experiments were performed to confirm their effect on CSCs.

ICPM suppresses breast cancer stem-like characters in vitro

To investigate effects of ICPM on the cancer stem-like cell population, we performed the mammosphere formation assay in human MCF-7 breast cancer cells. BCSCs have been demonstrated to be enriched in nonadherent spherical clusters of cells, termed mammospheres [15]. After treatment with varying concentrations of ICPM (0–6.25 μM) for 10 days, ICPM reduced the size of mammosphere concentration dependently (Fig. 8a); treatment for 21 days, ICPM markedly reduced both

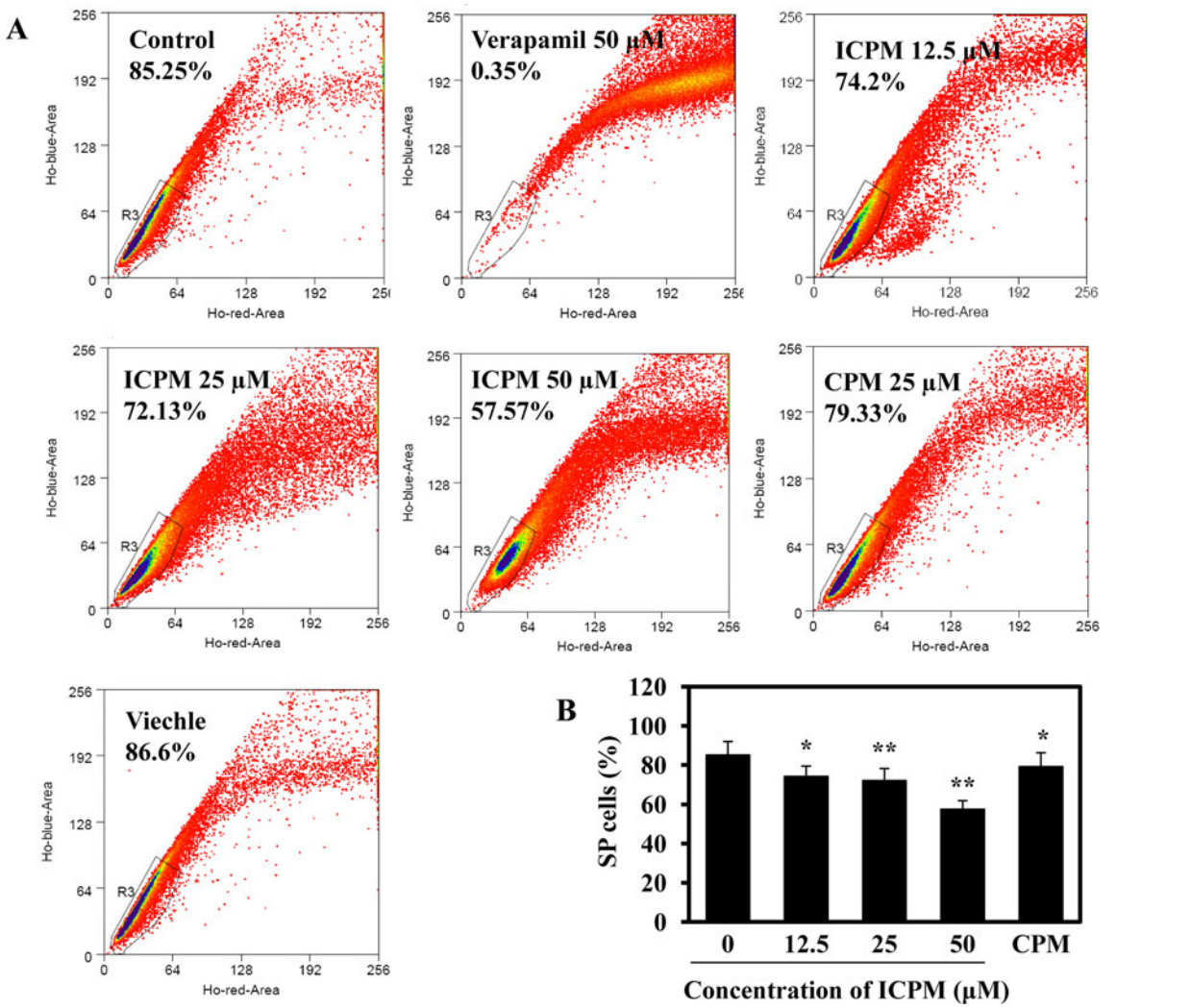

Fig. 5 ICPM-induced inhibition of SP cells in MCF-7/ADR cells. **a** A representative example of the SP proportion is displayed. After treatment with ICPM (12.5, 25, and 50 μM) for 48 h, SP cells were counted by flow cytometry using Hoechst 33342 staining. Verapamil, an inhibitor of ABC transporters, was used as a control to ensure the capture of SP cells by being used to set the gate. The SP was gated and shown as a percentage of the total cell population. **b** Histograms show the decrease percentage of SP cells. Data are shown as means ± SD of three experiments for each group. *$P<0.05$, **$P<0.01$ versus medium control

the size (Fig. 8b) and number of mammospheres (Fig. 8c), and CPM (6.25 μM) had a similar effect when compared with ICPM. These results suggested that ICPM could inhibit the formation of mammospheres.

Both $CD44^+$ and $CD24^-$ have been used as specific markers to identify the BCSCs from human tumor tissues [19]. The $CD44^+/CD24^-$ cell population is capable of self-renewal and generating tumors resembling breast cancer. To confirm the effect of ICPM on MCF-7/ADR stem-like cells, cells were stained by CD44-FITC and CD22-PE, after ICPM treatment for 48 h, and phenotype of $CD44^+/CD24^-$ cells was measured by flow cytometry. As shown in Fig. 8d, the $CD44^+/CD24^-$ cell percentage in the untreated MCF-7/ADR cells was 12.2 %; the percentage significantly decreased to 7.4 % when treated with 12.5 μM ICPM. Furthermore, the suppression on the cancer stem cell characteristics by ICPM was confirmed by the reduced expression of ALDH, which is another surface markers of cancer stem cells in solid tumors [20]. As shown in Fig. 8e, f, ICPM could time and concentration dependently decrease the level of ALDH. At the same concentration (25 μM), ICPM and CPM showed no obviously different effect. These findings therefore confirmed that ICPM could suppress the stem-like cell population in vitro.

Discussion

To overcome the metabolic stability issue (decomposition at pH<3) of CMP, analogs with an *exo*-methylene unit at C13–

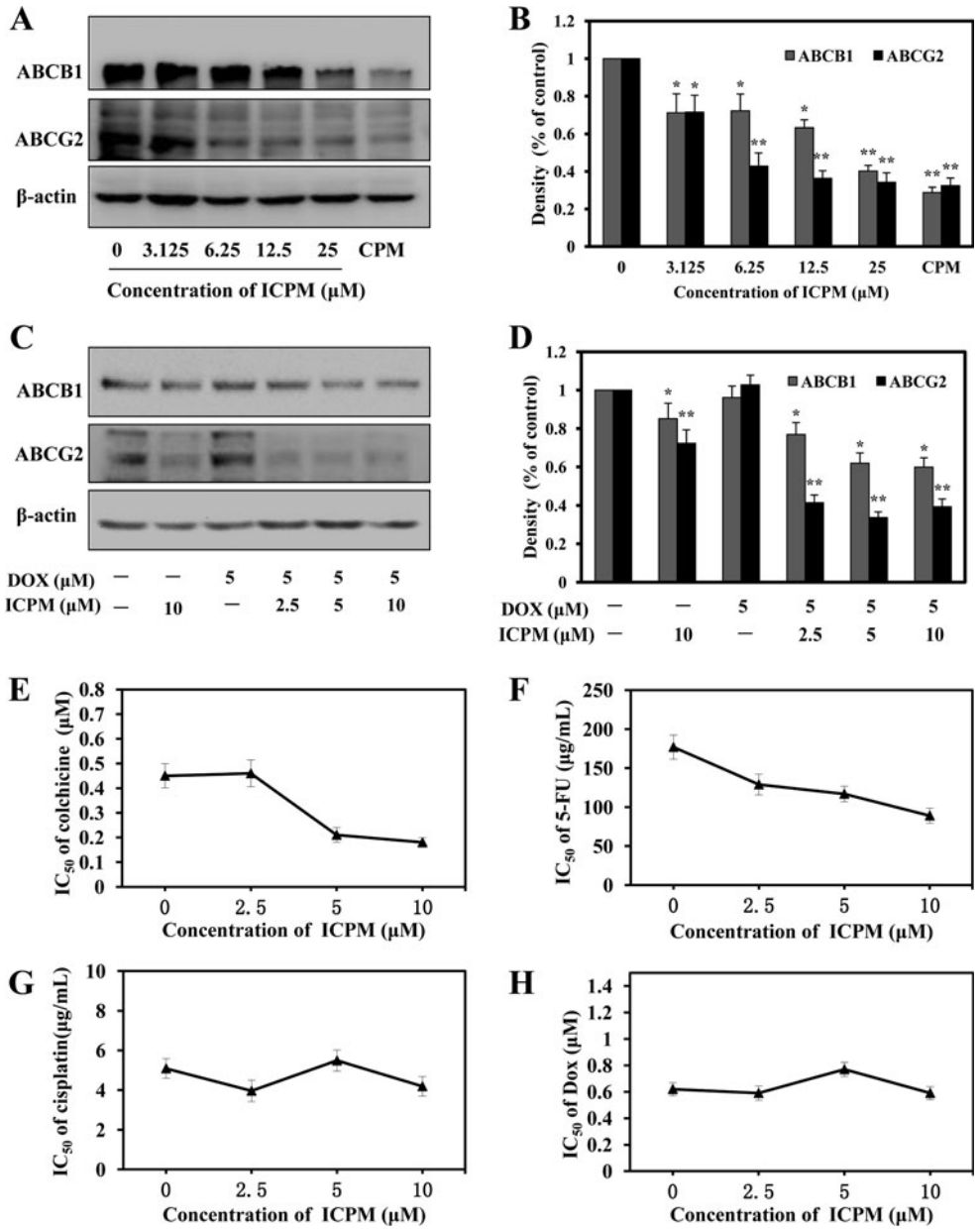

Fig. 6 ICPM decreases the protein levels and transport function of ABCB1 and ABCG2 in MCF-7/ADR cells. **a** Decreased expression of ABCG2 and ABCB1 in MCF-7/ADR cells. Cells were treated with certain concentration of ICPM (0–25 μM) and CPM (25 μM) for 48 h, and total cell lysate was obtained. Western blotting was performed as described in "Materials and methods". **b** Histograms show the decreased intensity of protein bands. Results are normalized to untreated cells, and the data shown are the means ± SD of three experiments for each group. *$P<0.05$, **$P<0.01$ versus medium control. **c** ICPM decreases the expression of ABCG2 and ABCB1 in MCF-7/ADR cells in the presence of DOX. Cells were treated with certain concentration of ICPM (0–10 μM) in the presence of 5 μM DOX for 48 h, and ABCB1 and ABCG2 were detected by Western blotting. **d** Histograms show the decreased intensity of ABCB1 and ABCG2 bands. Cells were treated with chemotherapy drugs for 72 h in the presence of 2.5, 5, and 10 μM ICPM, and the IC_{50} values were calculated, respectively. Growth inhibition was evaluated by MTT assay. ICPM reverses the multidrug resistance of ABCB1 substrate COL (**e**) and ABCG2 substrate 5-FU (**f**) but does not sensitize the cytotoxicity of cisplatin (**g**), which is non-ABCB1 and non-ABCG2 substrate. **h** ICPM does not sensitize the DOX in MCF-7 cells with low ABCB1 and ABCG2 levels

C18 (D ring) have been designed by Heretsch and his colleagues [11, 13] to increase the stability under acid conditions. This stability arises from the reduced overlap of the p-bonding orbital with the unoccupied p-orbital at C17, which decreases the opening of the furan ring in CPM [11]. In our synthesis course of CPM starting from jervine, we obtained a novel CPM derivative, ICPM, with the allylic double bond at position C11–C12. ICPM, with the double bond located at the C ring, was structurally different from the analogs bearing *exo*-methylene unit [11, 13] but had the same acid stability. Thus, ICPM was a new derivative of CPM that was more soluble and acid stable than CPM.

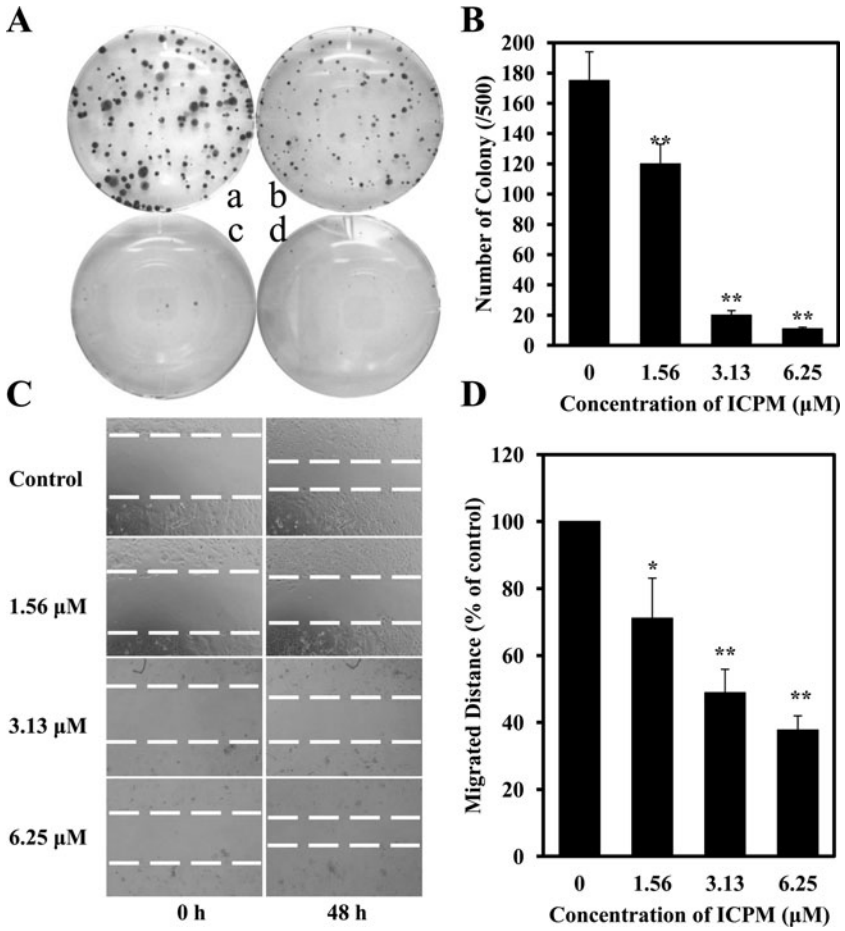

Fig. 7 ICPM decreases the colony formation and inhibits the migration of MCF-7/ADR. **a** The viability of MCF-7/ADR cells after ICPM and CPM treatment was determined by colony-formation assays. MCF-7/ADR cells were plated onto 6-well plates, at a density of 500 cells per well, ICPM (0–12.5 μM) and CPM (12.5 μM) was added and incubated for additional 16 days, cells were stained with Giemsa, and colonies were scored. Shown is a representative result from three experiments with similar results. **a** Control group; **b**, **c**, **d** is ICPM at 1.56, 3.13, 6.25 μM, respectively. **b** Histogram shows the number of colonies in the presence and absence of ICPM (0–6.25 μM). **c** ICPM inhibits the migration of MCF-7/ADR. Cells were treated without or with certain concentration of ICPM. After incubation for 48 h, cell migrations were analyzed using scratch-wound assay. **d** Quantitative evaluations of cell migration inhibited by ICPM in the scratch-wound assay. Results are normalized to untreated cells. All experiments were repeated more than three times. Values represent the means ± SD of triplicate measurements. $*P<0.05$, $**P<0.01$ versus medium control

The cytoxicity of ICPM in vitro was moderate and showed similar effect to CPM; however, ICPM showed a potent synergism with DOX in MCF-7/ADR cell lines. A series of experiments were conducted in this study to illustrate the mechanisms underlying the combination with commercial chemotherapeutics, in this case DOX. The intracellular concentration of DOX after co-incubation with ICPM was increased, indicating that the efflux of DOX was inhibited by ICPM. The increased intracellular accumulation of DOX was also observed in many other compounds, such as resveratrol [21] and SC236 [22], and this is one of the main mechanisms contributing to the enhancement of DOX cytotoxicity in drug resistance cells. A major contributor to the drug resistance is ABCB1, which acts as a drug efflux pump decreasing intracellular drug accumulation and therefore reduces intracellular drug efficacy [23]. A variety of compounds, such as cardiotonic steroids, have been screened and identified as new ABCB1 inhibitors [24]. Other research groups have observed that the downregulation of ABCB1 expression in DU-145 cells has a relationship with the reverse of the drug resistance [25]. In line with this, our present results revealed that ICPM could decrease the expression of ABCB1 in MCF-7/ADR cells, which is one of the mechanisms for the reverse of the DOX resistance. However, whether this compound acts as a direct inhibitor of ABCB1 ATPase needs more experimental evidences.

Conventional chemotherapeutic agents usually lead to an enrichment of SP, revealing their inability to target the drug-resistant SP and cancer stem-like cells. Thus, agents that can reduce the SP phenotype are currently in vogue in cancer therapeutics. The inhibition on the DOX efflux and the decrease in the level of ABCB1 made us wonder whether ICPM also regulated the SP cells, which overexpress detoxifying cell surface pump ABCG2. The SP strategy using cell-permeable

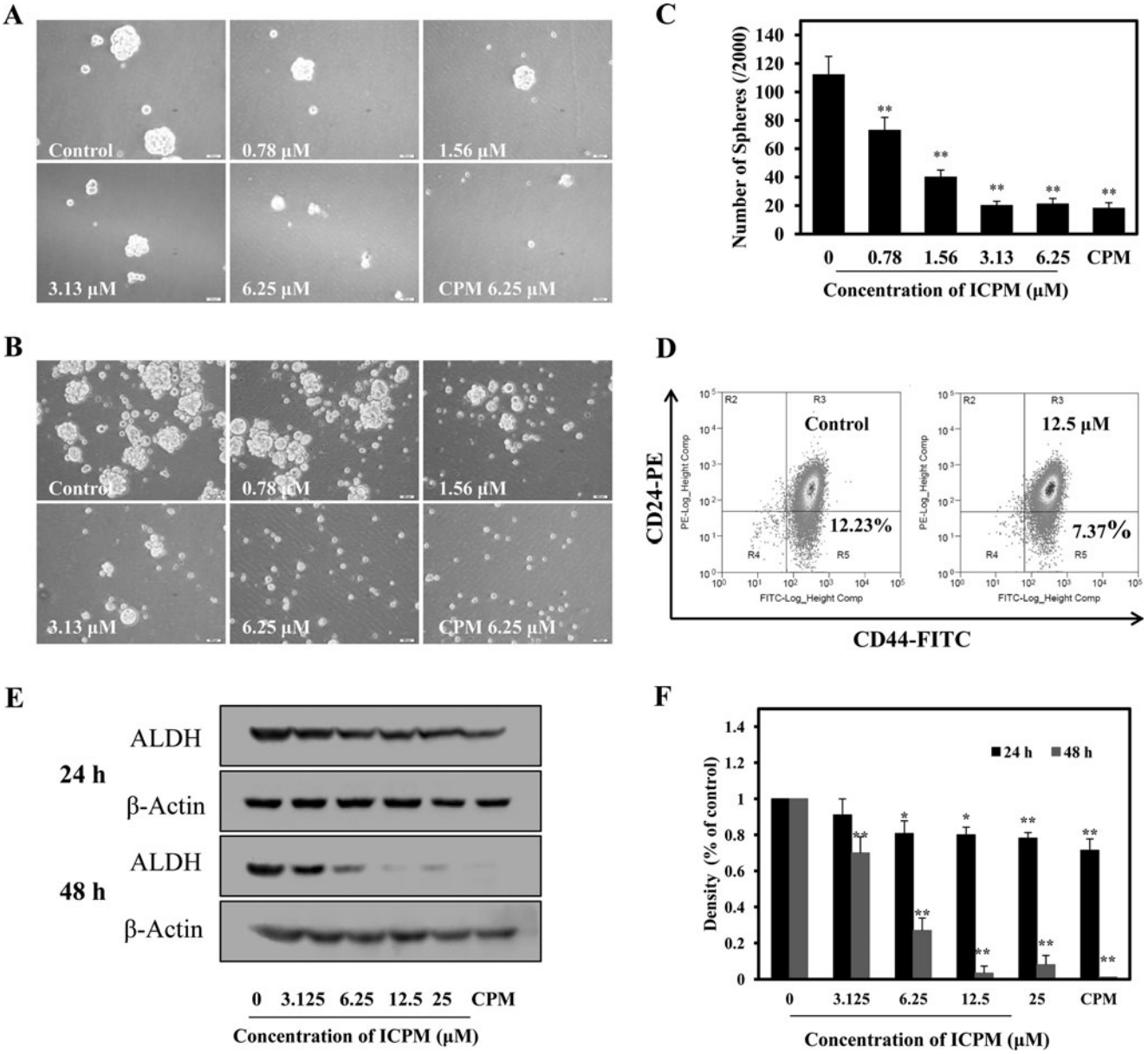

Fig. 8 ICPM inhibits cancer stem cell phenotypes in MCF-7/ADR cells. **a** ICPM decreases mammosphere formation in vitro. ICPM decreases the size of mammospheres (×200, *scale bar*=500 μm). MCF-7 cells were cultured in ultra-low-attachment plates with serum-free medium and treated with different concentrations of ICPM for 10 days. Mammospheres were observed and photographed under microscope. **b** ICPM decreases the size of mammospheres (×200, *scale bar*=500 μm) treated for 21 days. **c** The number of mammospheres is reduced when treated with ICPM for 21 days. Similar results were obtained in three independent experiments, and the representative microscopic pictures are shown. *$P<0.05$, **$P<0.01$. **d** ICPM reduces the percentage of $CD44^+/CD24^-$ breast cancer stem-like cells. The phenotype of $CD44^+/CD24^-$ cells was measured by flow cytometry after treatment with 12.5 μM ICPM for 48 h. **e** ICPM reduces the expression of cancer stem-like cell marker ALDH concentration and time dependently. The total level of ALDH in MCF-7/ADR cells was measured by Western blotting after treatment with ICPM and CPM for 24 and 48 h. **f** Histogram shows the decreased intensity of protein bands, which was quantified by ImageJ. Results are normalized to untreated cells, and the data are shown as means ± SD of three experiments for each group. *$P<0.05$, **$P<0.01$ versus medium control

dye Hoechst 33342 was then performed. As expected, ICPM decreased the SP percentage in MCF-7/ADR cells. Similarly, CPM had the same ability to regulate the SP cells in MCF-7/ADR cells as it does in other cell types [19]. Both the function and protein levels of ABCG2, which confers the SP phenotype, were reduced by ICPM and CPM, consistent with the previous finding that CPM downregulates efflux function of ABCG2 [19]. The modulation on ABCB1 and ABCG2 was further confirmed by the following evidence. ICPM could enhance the cytotoxicity of ABCG2 substrate 5-FU and ABCB1 substrate COL in MCF-7/ADR cells, rather than enhance the sensitivity of cisplatin, which is neither the substrate

of ABCG2 nor ABCB1. In addition, ICPM could not increase DOX sensitivity in MCF-7 cell line, which expresses low level of ABCG2 and ABCB1, indicating that ABCB1 and ABCG2 are indispensible for ICPM-induced reverse of the drug resistance.

ABCG2 acts as a vital regulator within CSCs, as well as a drug efflux pump. These CSCs are one of the reasons why most chemotherapy drugs eventually lose their efficacy. The SP cells possess stem-like properties, considering that ICPM had an obvious effect on the SP cells and the ABCG2 function, we next evaluated the effect of ICPM on the stem-like characteristics. Our results showed that ICPM suppressed proliferative, clonogenic, metastatic capacity, decreased mammosphere formation, reduced the level of CSC biomarker ALDH, and the percentage of $CD44^{+}/CD24^{-}$ cells, which are all characteristics of the CSCs. Therefore, the effect on the CSCs also contributed to the reverse of DOX resistance. In our present work, we found that although ICPM had the increased acid stability than CPM, the extent of its bioactivity was not much more potent than CPM, in the aspects of increasing the DOX accumulation and retention, downregulating the level of ABC transporters, as well as inhibiting the stem-like characteristics.

CPM is usually considered as an hedgehog (Hh) antagonist by direct targeting the product of the proto-oncogene Smoothened (Smo) [26]. ICPM, with highly similar structure to CPM, is also possibly an antagonist of Hh signaling pathway, which is one of the main pathways controlling the stem cells. Inhibition of Hh signaling pathway via direct interaction with Smo has been proved an efficient way [26, 27]. Smo is significantly elevated in tamoxifen-resistant MCF-7 cells, and anti-Hh compound blocks the tamoxifen-resistant xenografts tumor growth in mice [28]. All of these collected results made us wonder the effect of ICPM on MCF-7/ADR cells may via the inhibition of Hh signaling. We then investigated whether such ICPM-induced reverse in the DOX sensitivity had a relationship with Smo binding and inhibition of Hh signaling. Unexpectedly, we did not find the expression of Smo in MCF-7/ADR cell line when labeled with Smo-specific probe bodipy-cyclopamine and assayed by both flow cytometry and confocal microscopy (data not shown). Moreover, we did not find the expression of Gli in this cell line when detected by Western blotting, which lies downstream of Smo (data not shown). All of these clues indicated that activity of ICPM in this cell line was possibly Smo independent and not through regulating the Hh signaling.

It has been reported that ABC transporters also regulate stem cell signaling at the upstream [15]. This would be an alternate way to understand the complex of stem cell signaling and to illustrate compound regulation on this signal pathway. Previous research work showed that CPM possibly has a dual mode of action, inhibiting both Hh signaling and ABCG2-dependent efflux simultaneously and independently [19, 29], and interacts with ABCG2 directly [30], indicating ABCG2 is possibly a novel target of CPM. Furthermore, previous study showed that CPM inhibition of human breast cancer cell growth is also independent of Smo [31], indicating Smo is not the unique target of CPM and possibly other different targets are involved. From these collected results, we hypothesized that it was more likely that ICPM also inhibited the stem cancer cells via the regulation of ABC transporters while independent of Smo. However, we only investigated the function and expression level of ABC transporters' influence by ICPM; whether or not ABCG2 and ABCB1 had direct interaction with ICPM was not evaluated in this work, and further studies will be needed to discover how ICPM may also regulate these molecules directly.

In conclusion, we have successfully identified a new CPM derivative, ICPM, which showed better stability and solubility. Further insights were gained into the combination with DOX and ICPM, and we demonstrated that ICPM had the potent synergy with DOX. The mechanisms were related to the intracellular accumulation of DOX in the cells and the downregulation of the cancer stem-like cell characteristics. Taken together, ICPM will further contribute to its usefulness in biological and medicinal studies, as well as the development of anti-cancer drug with improved efficacy.

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Conflict of interest None

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