Isocorydine suppresses doxorubicin-induced epithelial-mesenchymal transition via inhibition of ERK signaling pathways in hepatocellular carcinoma

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Abstract: Doxorubicin (DOX) is a classical and effective chemotherapeutic used in the treatment of hepatocellular carcinoma (HCC). However, doxorubicin administration may induce EMT, which results in the development of chemoresistance in HCC. Recent studies report that Isocorydine (ICD) selectively inhibits human cancer stem cells (CSCs), which have an important role in the development of chemoresistance. In this study, we observed that ICD co-administration enhanced DOX cytotoxicity in HCC cells, enabling the inhibition of DOX-induced epithelial-mesenchymal transition (EMT). Microarray data analysis revealed substantially decreased ERK signaling after ICD treatment. Additionally, we observed decreased IC50 for DOX upon ERK knockdown. Finally, we confirmed the enhanced efficacy of treatment with a combination of DOX and ICD in xenograft models. Collectively, the present study unveils the benefit of using DOX in combination with ICD for chemotherapy against HCC, revealing a novel potential anti-cancer strategy.

Keywords: Doxorubicin, Isocorydine, HCC, EMT

Introduction

Hepatocellular carcinoma (HCC) is the fourth most commonly diagnosed human malignancies and third cause of cancer-related death in China [1]. Despite increased health examinations, most patients have either advanced cancer or metastases at the time of diagnosis [2]. High-grade carcinomas have a larger tumor volume and vasculature and surgery alone has low success rates. Patients who undergo surgical removal of cancer and are at a high risk of relapse must be considered for adjuvant therapy [3, 4]. While researchers have explored many strategies for chemotherapy in HCC treatment, the effective treatment choices remain limited. Resistance to conventional adjuvant therapy is a major problem for the effective treatment of cancer [5, 6]. Hence, screening for agents that improve the chemotherapeutic efficacy of HCC is valuable to sensitize chemoresistant tumors.

Doxorubicin (DOX) is a classical antineoplastic agent that has been widely used in HCC treatment [4]. Previously, we have reported that DOX administration induces Epithelial-Mesenchymal Transition (EMT) in HCC [7]. During EMT, epithelial cells transform into motile, invasive mesenchymal cells and play an important role in the progression of malignant tumors, including tumor proliferation, invasion and migration. Previous studies have demonstrated that EMT can confer cancer stem cell-like properties [8]. EMT process in cancer cells may further promote chemoresistance in many malignancies, including pancreatic cancer [9], breast cancer [10] and HCC [7]. However, the precise mechanism of DOX-induced EMT remains ambiguous.

L-(+)-isocorydine (ICD) extract from Dicranostigma leptopodum (Maxim.) Fedde (DLF) is an active ingredient in DLF that significantly inhibits the cell proliferation of HCC side population (SP) in vitro and in vivo [11]. Side population cells, defined as a subpopulation of tumor cells with the capacity for self-renewal and tumor initiation and are an important attribute of cancer stem cells (CSC). We postulate that ICD has the
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ability to inhibit CSC associated development of chemoresistance in HCC cells when used in combination with DOX.

In this present study, we investigated the selective mechanisms involved in ICD mediated inhibition of DOX-induced EMT. We further report the important role of ERK signaling pathway in the inhibition of HCC cell growth after a combined treatment with DOX and ICD.

Materials and methods

Cell lines and cell culture

Human HCC cell lines (SNU-449, SNU-387, Huh-7, and Hep-G2) were procured from the Shanghai Institute for Biological Science, China. All cell lines were cultured in DMEM medium (high glucose; Gibco) containing 10% fetal bovine serum (FBS; Gibco) supplemented with 1% penicillin/streptomycin (Sigma, St. Louis, MO). Cells were maintained at 37°C in a humidified incubator with 5% CO₂.

Doxorubicin was purchased from Merck KgaA (Darmstadt, Germany). Isocorydine was purchased from Herbest Company (Shanxi, China).

Cell viability assays

Cell toxicity and proliferation after treatment were determined using the cell counting kit-8 assay (CCK8; Dojindo, Kumamoto, Japan) according to the manufacturer’s specifications. Briefly, 5000 hepatoma cells were seeded per well in a 96-well microplate and exposed to different concentrations of the drug for 48 h. 10 μl of CCK8 reagent (in PBS) was added to each well and incubated for another 4 h, followed by

Figure 1. ICD enhances DOX inhibition of HCC proliferation in CCK-8 assay. Relative cell viability (mean ± SD) for ICD (black), DOX (red) and ICD + DOX (blue) in Huh-7, HepG2, SNU-449 and SNU-387 cells.
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measurements of optical density at 450 nm. The half-maximal inhibition concentration (IC₅₀) was calculated as described previously [12]. All experiments were conducted in triplicates.

Western blotting and immunofluorescence

Cells were treated with drugs for 48 h and lysed in lysis buffer, followed by separation of proteins by 10% SDS-PAGE. The proteins were transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). Anti-E-cadherin, anti-Vimentin, anti-Twist1 antibodies (1:1000; Cell Signaling) were used to detect the respective proteins. HRP conjugated secondary antibodies were detected by chemiluminescence using ChemiDoc MP system (Bio-Rad, Hercules, CA). GAPDH was used as a loading control.

Cultured cells were maintained in a chamber slide (Millipore, Billerica, MA) for 24 h. Cell were fixed with 4% paraformaldehyde for 30 min at room temperature. The slides were washed in PBS and non-specific sites were blocked with 5% BSA for 30 min. The slides were incubated overnight at 4°C with mouse anti-human Vimentin or anti-human E-cadherin primary antibody (1:1000; Cell Signaling). After washing in PBS, the slides were incubated with PE or FITC conjugated secondary antibody for 1 h, followed by washing in PBS. DAPI was used to stain the nuclei. Stained slides were observed under confocal microscope (Carl Zeiss, Oberkochen, Germany).

Wound healing assay

The cells were cultured in the medium supplemented with or without the half-maximal inhibitory concentration (IC₅₀) of indicated drugs. Same cell numbers were seeded in 12-well plates pre-coated overnight with 0.5% gelatin. Once confluent, the cells were starved in 1% FBS culture medium for 24 hours. Using a 200 µl pipette tip was used to create a scratch wound on the cell monolayer. The distance migrated by the cells was calculated as an average of three different fields observed under optical microscope. Three independent experiments were analyzed at 200x magnification.

Transwell invasion assay

The Transwell inserts were coated with Matrigel (BD Biosciences, Bedford, MA, USA). Equal number of cells in each upper chamber were cultured in medium supplemented with 1% FBS. The lower chamber was supplemented with 20% FBS medium as a chemoattractant. Cells were incubated for an additional 24 hours at 37°C in 5% CO₂. Cells in the lower chamber of the Transwell filter were fixed with 95% ethanol for 15 min followed by hematoxylin and eosin staining. Once stained, the cells were evaluated under the microscope.

Flow cytometry and sorting

CD 133 staining in HCC cells was performed using fluorochrome-conjugated mAbs with parallel controls (Biolegend, San Diego, CA). Cell suspension containing 1×10⁶ cells/mL was stained with CD133 at 4°C for 30 min. Flow cytometric analysis was performed on a Canto-II (BD Biosciences, San Jose, CA). CD133+ or CD133- cells were sorted using FACSAria II (BD Biosciences, San Jose, CA).

Xenograft experiments

Animal experiments were conducted in compliance with the Guide for the Care and Use of the Animal Ethics Committee of Wenzhou Medical University (Wenzhou, PR China). Female nude mice (Shanghai, China) aged 3-4 weeks were used for all experiments. 1×10⁶ Huh-7 cells were suspended in 100 µl PBS and implanted subcutaneously into the right axillary fossa of each mouse. Mice were randomly assigned to four subgroups including: DOX (4 mg/kg), ICD

<p>| Table 1. IC₅₀ values for DOX and ICD in HCC cells |
|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th><strong>Cell Line</strong></th>
<th><strong>IC₅₀ of ICD (µg/ml)</strong></th>
<th><strong>IC₅₀ of DOX (µg/ml)</strong></th>
<th><strong>Combination Index</strong></th>
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<tbody>
<tr>
<td>Huh-7</td>
<td>161.3</td>
<td>91.41</td>
<td>0.6565</td>
</tr>
<tr>
<td>Hep-G2</td>
<td>148</td>
<td>90.45</td>
<td>0.8796</td>
</tr>
<tr>
<td>SNU-449</td>
<td>262.2</td>
<td>178.3</td>
<td>1.739</td>
</tr>
<tr>
<td>SNU-387</td>
<td>254.1</td>
<td>161.5</td>
<td>2.353</td>
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*IC₅₀ of ICD concentration in different treatments for 48 h; **IC₅₀ of DOX concentration in different treatments for 48 h. *P < 0.05, **P < 0.01, ***P < 0.001.
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(0.4 mg/kg) and DOX (4 mg/kg) combined with ICD (0.4 mg/kg), as well as one vehicle-treated control group (equal volume of diluents). Drugs were injected intraperitoneally every 2 days for 2 weeks.

**Statistical analysis**

All data are presented as mean ± standard deviation (SD). The inhibitory effects of different groups were compared using two-way (ANOVA), followed by Bonferroni post-hoc test. Student’s t-test was used to compare continuous variables and Chi square test (or Fisher’s exact test) was used to compare categorical variables. All tests are two sided and \( P < 0.05 \) was considered to be statistically significant.

**Results**

*ICD enhances DOX sensitivity in hepatoma cells*

To investigate if there was a dose-dependent effect of ICD on DOX mediated cytotoxicity in HCC cells, cell viability was measured 48 h after treatment using a CCK8 assays. As shown in **Figure 1**, combined treatment with ICD and DOX had a higher cytotoxicity in HCC cells in comparison to ICD or DOX alone. This is accompanied with a considerable decrease in IC\(_{50}\) for DOX. To further investigate the dose dependent relationship of adding ICD to DOX in HCC cells, the IC\(_{50}\) and combination index (CI) was calculated according to the previous study [12]. As shown in **Table 1**, a significant decrease in the IC\(_{50}\) for DOX is observed post combined treatment with ICD. The CI values were 0.605, 0.644, 0.804, and 0.707 respectively for Huh-7, Hep-G2, SNU-449 and SNU-387, indicating that the addition of ICD chemosensitizes HCC cells to DOX.

*ICD inhibits DOX-induced EMT*

Following treatment with DOX for 48 h, we observed an upregulation of Vimentin, β-catenin and Snail. However, protein levels of Claudin-1 and E-cadherin were downregulated (**Figure 2A, 2B**). The cell morphology changed upon...
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A  

Huh-7  
Control  DOX  DOX+ICD  ICD  

Hep-G2  

B

Huh-7  
Ctrl  DOX  DOX+ICD  ICD  

Hep-G2  

C

Huh-7  
Ctrl  DOX  DOX+ICD  ICD  

Hep-G2  

D

Huh-7  
Ctrl  DOX  DOX+ICD  ICD  

Hep-G2  

E

Huh-7  
ICD  DOX+ICD  DOX  Ctrl  

Hep-G2  
ICD  DOX+ICD  DOX  Ctrl  

F

Huh-7  
CD133  

Hep-G2  
CD133  

G

CD133+ Huh-7  

CD133+ Hep-G2
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Figure 3. ICD inhibits the DOX-induced tumor invasive capacity in HCC. (A) Transwell assays showing the invasion of Huh-7 and Hep-G2 cells for control, DOX, DOX + ICD or ICD group (100x magnification). (B) Number of cells per field that crossed the transwell chamber under the different treatment conditions (P < 0.05, **P < 0.01, ***P < 0.001). (C) Wound healing assay for migration of Huh-7 and Hep-G2 cells in the control, DOX, DOX + ICD or ICD group (100x magnification). The broken white line indicates the wound areas without migrating cells. (D) The gap between the migrating cells is expressed as a percentage of the initial area. (E) Levels of CD133 in HCC cells (Huh-7 and Hep-G2), or HCC cells treated with DOX, DOX + ICD, or ICD alone for 48 h. Sorting CD133 + HCC cells (F) and the relative cell viability for ICD (black), DOX (red) and ICD + DOX (blue) in Huh-7 and Hep-G2 (G). One of three representative images are shown in (A, C).

Table 2. IC50 values for DOX and ICD in CD133+ HCC cells

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>IC50 of ICD (μg/ml)†</th>
<th>IC50 of Dox (μg/ml)‡</th>
<th>Combination Index</th>
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<tbody>
<tr>
<td>Huh-7</td>
<td>207.1</td>
<td>69.72**</td>
<td>0.456</td>
</tr>
<tr>
<td>Hep-G2</td>
<td>370.4</td>
<td>129.9**</td>
<td>0.556</td>
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*IC50 of ICD concentration in different treatments for 48 h; †IC50 of DOX concentration in different treatments for 48 h. **P < 0.01, ***P < 0.001.

treatment with DOX to a stretched, elongated shape. Furthermore, immunostaining experiments revealed that DOX exposure induced a strong cytoplasmic localization of Vimentin and weak membrane expression of E-cadherin (Figure 2C, 2D), indicating that treatment with DOX resulted in the acquisition of EMT-like characteristics by HCC.

We next investigated the effect of ICD on DOX-induced EMT after co-treatment of HCC with DOX and ICD or DOX alone for 48 h. ICD abrogated DOX-induced upregulation of mesenchymal markers and the downregulation of epithelial markers (Figure 2A, 2B). Furthermore, immunofluorescence staining showed a weaker signal for Vimentin and strong E-cadherin staining in co-treated cells (Figure 2C, 2D). These results demonstrate that DOX-induced EMT in epithelial cells was inhibited by ICD.

ICD inhibits the invasive capacity of DOX-induced EMT in HCC cells

Transwell assays showed remarkably less hepatoma cells passing through the matrigel coated membranes when compared with the corresponding cells under single drug condition (Figure 3A, 3B). Wound healing assays also showed decreased migration in the cells exposed to combined treatment with ICD and DOX, when compared to the cells treated with DOX or ICD alone (Figure 3C, 3D). These results suggest that addition of ICD decreased the invasiveness of DOX-induce EMT in HCC cells.

The proportion of CD133 positive cells was markedly elevated in the cells treated with DOX. This phenotype disappeared after the combined treatment with DOX and ICD (Figure 3E). To further test the inhibition of CD133+ HCC cells, we sorted CD133+ cells (Figure 3F). CCK8 assay confirmed higher resistance of CD133+ cells to chemotherapeutic agents (Figure 3G). However, this subpopulation of CD133+ cells was sensitive to the combined treatment with ICD and DOX. The CI value were 0.456 and 0.556 respectively for Huh-7 and Hep-G2 (Table 2). These results suggest that the stem cell-like subpopulation of CD133+ cells was sensitive to the combined treatment with ICD and DOX.

ICD inhibits EMT induced by DOX by inhibiting ERK signaling

Next, we used microarray analysis to gain specific insight into the mechanism of inhibition of EMT by ICD. Genes whose expression levels showed a ≥ 2-fold change in control vs. DOX-treated and DOX-treated vs. DOX + ICD-treated group were identified. Data showed that ICD significantly inhibits the DOX-induced EMT (Figure 4A). Following an overlay analysis, 502 genes were identified and clustered (Figure 4B). 101 genes were associated with specific signaling pathway highlighted by GO analysis (Figure 4C). A relevant percentage of these genes i.e. ELK1, ELK4 and MYC (Figure 4D) were correlated with mitogen-activated protein kinase (MAPK) signaling, indicating the role of MAPKs in ICD mediated reversal of DOX-induced EMT.

We further investigated the involvement of the MAPK pathway in DOX induced EMT and found that treatment with DOX increased the levels of p-ERK expression in comparison to the other groups. However, the levels of p-ERK decreased after the co-treatment with ICD (Figure 5A). To confirm the involvement of the ERK pathway, we used siRNA to knockdown p-ERK1/2 expression. Knockdown of p-ERK significantly attenuated...
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The EMT transition also results in the increased capacity for migration and invasion. The EMT process may be responsible for chemoresistance and invasion in hepatoma, leading to metastatic events [13-15]. Doxorubicin is an effective chemotherapeutic agent for TACE, and has been shown to induce EMT in HCC in our previous study [7]. Doxorubicin-

Figure 5. ICD reverses DOX-induced EMT through ERK signaling. A. Expression of ERK1/2 examined by western blot in Huh-7, Hep-G2, or treated with DOX, DOX + ICD, or ICD alone for 48 h. B. Detection of EMT markers upon treatment with ERK1/2 siRNA. D. Immunofluorescence staining of E-cadherin and Vimentin in DOX and DOX + ERK siRNA in Huh-7 and Hep-G2 (200x magnification). C. The relative cell viability for DOX (red) and DOX + ERK siRNA (black) in Huh-7 and Hep-G2.
Isocorydine reverses doxorubicin-induced chemoresistance

Isocorydine (ICD) is an alkaloid monomer derived from Papaveraceae spp. plant, including Dactylicapnos scandens Hutchins and Dicranostigma leptopodum (Maxim) Fedde (DLF). ICD, which is the active ingredient in DLF, is known to induce apoptosis in HCC cell lines and the side population (SP) cells [11]. Previous results indicate that ICD may target CSC-like cells [11]. In the present study, we evaluated the synergistic inhibitory effect of ICD in HCC cell lines when co-administered with DOX. ICD remarkably decreased the expression of DOX induced CD133. Furthermore, ICD reversed DOX-induced changes in the expression of EMT-markers and attenuated the invasion and migration ability after treatment with DOX.

The transcription factors involved in EMT can typically be classified into three protein families: the Snail family (including Snail and Slug), ZEB family (including ZEB1 and ZEB2), and basic helix-loop-helix (including TWIST1, TWIST2, and TCF3) family [19]. The transcription

induced EMT may substantially decrease the chemotherapeutic efficacy and lead to distant metastasis. However, there is no systematic study investigating the role of signaling events resulting in DOX resistance.

Accumulating studies indicate that activation of the EMT is necessary, not only for the physical dissemination of tumor cells, but also for the transformation to the CSC state, enabling metastasis of the cells disseminated from the primary tumor, and development of resistance to the chemotherapeutic agents [16-18]. EMT also results in the production of several autocrine signaling loops, including the transforming growth factor β (TGFβ) and Wnt pathways. These positive signaling loops contribute further to the acquisition of CSC properties in the cells [13]. We found that DOX-treated cells substantially increase the expression level of CD133 and therefore presumed that a combination of CSC-target drug may provide a viable strategy to tackle DOX-resistance.

Figure 6. Effect of ICD, DOX, and their combination in nude mice implanted with subcutaneous HCC xenografts. A. Tumor volume over time in the control group (black), groups treated with DOX (blue), ICD (green) or DOX + ICD (red). Relative tumor volume ratios (% of original volume after initiation of therapy) are presented as the mean ± SD, (**P < 0.01, ***P < 0.001, for control vs. DOX group; #P < 0.01, ###P < 0.001, for DOX + ICD vs. DOX alone). B. Resected tumors in the different groups. C. The body weight of mice after treatment.
factor ZEB1 is involved in the EMT process, repressing the expression of E-cadherin, which can be activated by the TGF-β, TNF-α and IGF1 signaling pathways [20]. A correlation between ZEB1 expression and loss of E-cadherin has been confirmed in lung adenocarcinomas [21]. ERK induces EMT via regulation of ZEB protein expression [22]. Our data from the microarray assay and western blot analysis suggest that MAPK cascade is also involved in EMT. In addition, we found that the knockdown of ERK1/2 significantly inhibits ZEB-1 levels. Based on our results, we propose that ICD inhibits the phosphorylation of ERK1/2 and ZEB-1 expression resulting in the reversal of DOX-induced EMT.

In conclusion, our study provides evidence for the development of a potential DOX based anti-tumor chemotherapy to counter the issue of chemoresistance in HCC. However, further studies evaluating the role of specific transcription factor(s) regulated by the ERK cascade in ICD treatment are mandated for the comprehensive understanding of the development of chemoresistance in HCC.

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Disclosure of conflict of interest

None.

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Isocorydine reverses doxorubicin-induced chemoresistance


