Reversal of P-gp-mediated multidrug resistance in colon cancer by cinobufagin

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Abstract. Cinobufagin (CBF) is isolated from the skin and posterior auricular glands of the Asiatic toad (Bufo gargarizans). This study investigated the reversal effect of CBF on P-glycoprotein (P-gp)-mediated multidrug resistance (MDR) in colon cancer. The effect of CBF on the cytotoxicity of anti-cancer drugs in P-gp overexpressing LoVo/ADR, HCT116/L, Cao-2/ADR cells and their parental cells was determined using CCK-8 assay. Apoptosis of anti-cancer drugs and accumulation of doxorubicin (DOX) and Rhodamine 123 (Rho123) in P-gp overexpressing cells were evaluated by flow cytometry. Results indicated that CBF significantly enhanced the sensitivity of P-gp substrate drugs on P-gp overexpressing cells, but had no effect on their parental cells. CBF enhanced the effect of DOX against P-gp-overexpressing LoVo/ADR cell xenografts in nude mice. Moreover, CBF also increased cell apoptosis of chemotherapy agents and intracellular accumulation of DOX and Rho123 in the MDR cells. Further research on the mechanisms revealed non-competitive inhibition of P-gp ATPase activity, but without altering the expression of P-gp. These findings demonstrated that CBF could be further developed into a safe and potent P-gp modulator for combination use with anticancer drugs in cancer chemotherapy.

Introduction

Colon cancer is a common malignant tumor in the clinic, and its mortality is ranked third of all cancers. The incidence of colon cancer has been increasing over the past few decades (1). The main causes of failure of the treatment are postoperative metastasis and drug resistance to chemotherapy drugs (2).

Multidrug resistance (MDR), is the major cause for chemotherapy failure and it happens when cancer cells resist simultaneously to multiple chemotherapeutic agents, which are both structurally and functionally unrelated (3). The mechanisms of MDR include increased efflux of drugs, decreased uptake of drugs, impaired apoptotic pathways, and altered cell cycle checkpoints. The overexpression of ATP binding cassette (ABC) membrane transporter proteins that actively pump anti-cancer drugs out of the cells is the most important mechanism for MDR (4). Three major ABC transporters, i.e., ABCB1 (P-glycoprotein/P-gp), ABCC1 (multidrug resistance-associated proteins/MRP1), and ABCG2 (breast cancer resistance protein/BCRP), are commonly observed in cancer cells and critical to mediate MDR (5).

P-gp, a 170-kDa transmembrane glycoprotein encoded by the MDR1 gene, is the most studied member of ABC transporter family. It is extensively distributed in intestinal epithelium, hepatocytes, and kidneys, which is responsible for protecting tissues from a variety of toxins and xenobiotics (6). P-gp is also overexpressed in cancer cells, which can cause MDR and chemotherapy failure caused by reduction of the concentration of anti-tumor drugs within the cells (7). To date, three generations of P-gp inhibitors have been developed, such as verapamil, quinine, dexverapamil, emopamil, valspodar, and tariquidar (8). However, few of these inhibitors have progressed beyond clinical trials due to them exhibiting non-specific toxicity. Therefore, it is necessary to explore novel P-gp inhibitors with improved specificity and higher potency. A large number of traditional Chinese medicines have been observed to present potent anti-cancer activities, and some have become promising candidates as potential P-gp reversing agents (9,10).

ChanSu, a traditional Chinese medicine, derived from the skin and postauricular glands of the Asiatic toad (Bufo *gargarizans*) has been widely and successfully used for centuries for analgesia, and in the treatment of inflammation and cardiac arrhythmias (11). Moreover, it is also used to treat various cancers, such as colorectal, liver, and lung cancer in China (12). Cinobufagin (CBF) is one of the principal bioactive components of ChanSu, which is a traditional

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Chinese medicine. CBF is a major digoxin-like, bufadienolide steroid isolated from ChanSu, which has been reported to exhibit significant antitumor activity with the mechanism of inhibiting cell proliferation, inducing cell differentiation and apoptosis (13-15). Zhang *et al* reported that CBF suppressed tumor growth through intrinsic mitochondria apoptosis via AKT signaling pathway in human non-small cell lung cancer cells (16). However, very little is known concerning the role of CBF in circumvention MDR in colon cancer. Our group previously investigated ChanSu and its active ingredients (17-19) indicating that CBF can reverse chemoresistance of cancer cells, but its mechanism was not clear. In this study, we investigated the role and mechanism of CBF on reversing P-gp mediated MDR both *in vitro* and *in vivo*.

Materials and methods

Materials. CBF was purchased from Chengdu Herbpurify Co., Ltd. (Sichuan, China). Doxorubicin (DOX), Rho123, verapamil and Lucifer yellow were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Oxaliplatin (L-OHP) was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Minimum Essential Media (MEM), fetal bovine serum (FBS), non-essential amino acids (NEAA), Ham's F-12K (Kaighn's) medium (F12K), and Hank's balanced salt solution (HBSS), were obtained from Gibco BRL (Carlsbad, CA, USA). RPMI-1640 and phosphate-buffered saline (PBS) were from Hyclone (Thermo Scientific, Logan, UT, USA). Annexin V-FITC Apoptosis Detection kit was from BD Biosciences (Beijing, China). A P-gp ATPase assay system was purchased from Promega (Madison, WI, USA). The primary antibodies for caspase-3, caspase-9, P-gp and β-actin were from Cell Signaling Technology (Boston, MA, USA). The secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo (Kumamoto, Japan).

Cell lines and culture conditions. LoVo, Caco-2, and HCT116 human colorectal carcinoma cells were obtained from the Cell Bank of the Chinese Academy of Sciences. LoVo cells were cultured in F12k medium. Caco-2 cells were cultured in MEM medium, and HCT116 cells were maintained in RPMI-1640 medium. P-gp-overexpressing HCT116/L-OHP (HCT116/L) cells were established by our laboratory (20). L-OHP (5 μg/ml) was added to the medium of HCT116/L cells to maintain resistance, and then incubated in drug-free medium for minimum one week before use. P-gp-overexpressing LoVo/ADR and Caco-2/ADR cells were obtained from Shanghai Yan Sheng Industrial Co., Ltd. LoVo/ADR, HCT116/L and Caco-2/ADR cells were cultured in RPMI-1640 medium. DOX (8 μg/ml) was added to the medium of LoVo/ADR or Caco-2/ADR cells to maintain resistance and incubated for minimum one week in drug-free medium before use. All of the above cells lines were grown in culture flasks or dishes with medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in an atmosphere of 5% CO_2 and 90% relative humidity at 37˚C.

Cell cytotoxicity by CCK-8 assay. Cell viability was determined by CCK-8 assay (21). In brief, the cells were seeded at $1x10^5$ cells/ml in 96-well plates and were incubated overnight. A range of different concentrations of chemotherapeutic drugs, with or without verapamil (20 μM) or CBF, was added to the plates and incubated at 37˚C. After 48 h of incubation, 10 μl CCK was added to the each well and the plates were incubated for 1-4 h. The optical density was measured at 450 nm by Thermo Varioskan Flash (Thermo Scientific, Waltham, MA, USA). The degree of resistance was estimated by dividing the IC_{50} for the drug-resistant cells by that for the sensitive parental cells; the fold-reversal factor (RF) of MDR was calculated by dividing the IC_{50} values of the anticancer drug obtained in the absence of CBF by those obtained in the presence of CBF.

Apoptosis detection assay. The cell apoptosis rate of LoVo/ADR, HCT116/L and Caco-2/ADR cells was measured by flow cytometry using the Annexin V/PI Apoptosis Detection kit in accordance with the manufacturer's protocols. Cells were seeded at $1x10^5$ cells/ml onto 6-well plates and cultured with CBF, DOX and CBF+DOX for 48 h. After cells were trypsinized and collected into 5 ml tubes in 500 μl of 1X binding buffer, 5 μl PI and 5 μl Annexin V-FITC were added to each samples. Then samples were analyzed (FL-1:E_x= 488 nm, E_m= 530 nm; FL-2:E_x= 488 nm, E_m=620 nm) by FACS (BD Biosciences, San Jose, CA, USA).

Doxorubicin and Rho123 accumulation assay by flow cytometry. The intracellular accumulation of Dox and Rho123 in LoVo/ADR, HCT116/L and Caco-2/ADR cells was measured by flow cytometry as previously described (22). First, the cells were plated onto 6-well plates at a density of 10^5/well and were then incubated with CBF or verapamil for 48 h. Then cells were exposed to Dox (5 μg/ml) and Rho123 (1 μg/ml) at 37˚C for 90 min. After treatment, cells were trypsinized and collected, washed three times with cold PBS, and analyzed by FACS (BD Biosciences). Verapamil was used as a positive control.

P-gp-mediated drug transport assay. The transport experiments were performed using a method described previously (23). In brief, Caco-2 cells were seeded onto permeable polycarbonate filter inserts in 12-well transwell plate (0.4 μm pore size, 1.13 cm^2 of growth area, 12-mm diameter, Corning Costar Co., Corning, NY, USA) at a density of $1x10^5$ cells/ml and were allowed to grow for 21 days. The integrity of the monolayer was monitored by detecting the transepithelial electrical resistance (TEER) and Lucifer yellow permeability. Caco-2 monolayers with Lucifer yellow permeability <1% and TEER values >250 $\Omega \cdot cm^2$ were considered intact and used for the transport studies. The experiment was carried out in HBSS solution (pH 7.4). The monolayer cells were washed three times with HBSS and equilibrated for approximately 15 min. DOX (20 μM) was then added to the apical (A) or basolateral (B) side, and samples (0.1 ml) were taken at different time points (0, 15, 30, 45, 60, 90, 120 min) from the other side. Equal volumes of blank buffer were supplied after each sample withdrawal during the experiment. The concentrations of DOX were analyzed by Thermo Varioskan Flash.

P-gp ATPase assay. The changes of ATPase activity were measured by Pgp-Glo™ assay systems (24). In brief,

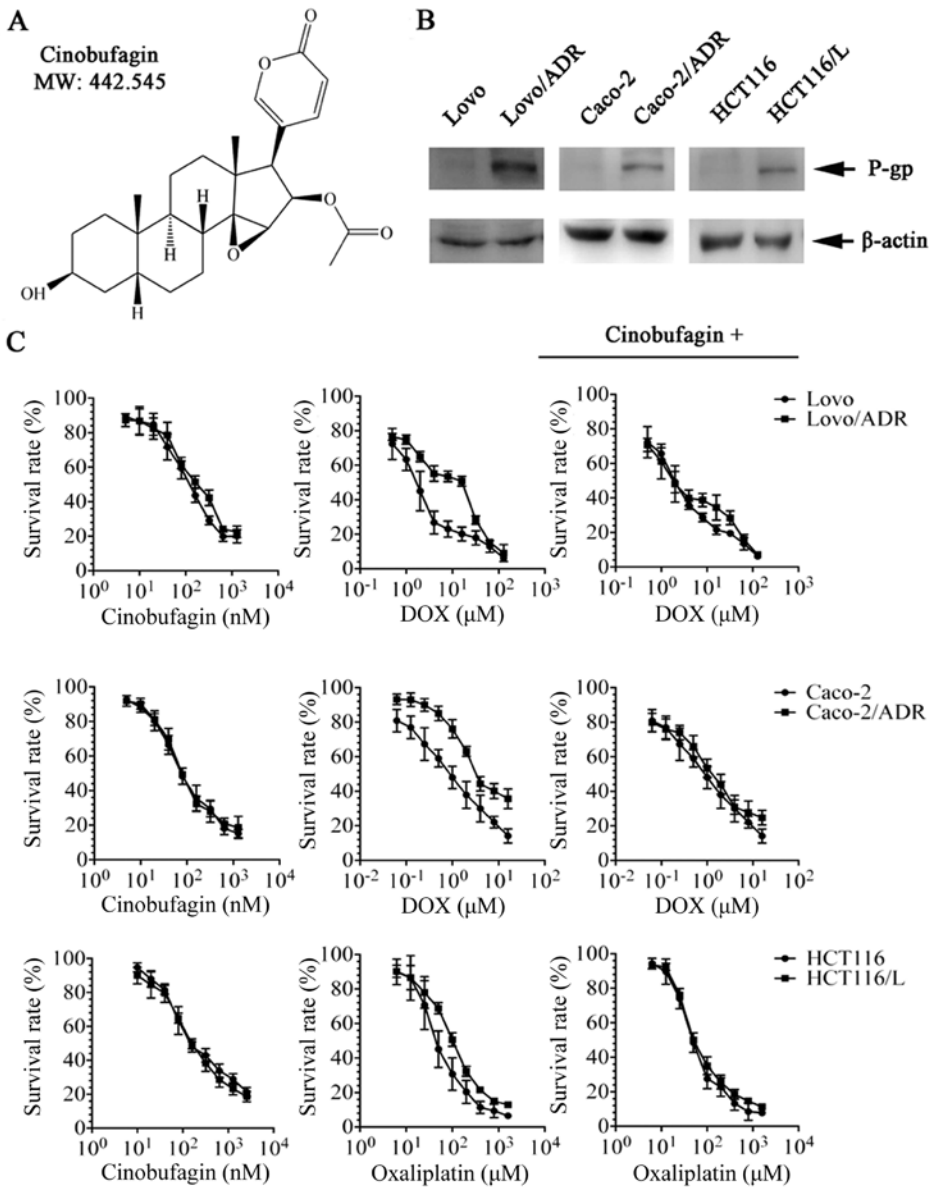

Figure 1. Cytotoxicity of cinobufagin in P-gp-overexpressing and parental cell lines. (A) Chemical structure of cinobufagin. (B) Western blot analysis of P-gp in drug-resistant cell lines and parental cells. β-actin was used as a loading control. (C) CCK-8 assay was used to evaluate cytotoxicity of cinobufagin in pairs of MDR and parental cell lines (left column); cytotoxicity of chemotherapy drugs (Dox or oxaliplatin) in pairs of MDR and parental cell lines (middle column); cytotoxicity of Dox or oxaliplatin in the presence of cinobufagin in pairs of MDR and parental cell lines (right column). Representative curves are shown as cell survival rate versus concentration of compounds. Error bars represent the SD.

samples containing verapamil (0.20 mM) and CBF (5, 10, 20, 50, 100 nM) was cultured with recombinant human P-gp membranes in untreated, white, opaque, 96-well plates (Inc., Lowell, MA, USA) for about 5 min at 37˚C. Reactions were initiated by adding 10 μl of 25 mM MgATP to all wells, and then the wells were incubated at 37˚C for 40 min on a heat block. Subsequently, the reaction was terminated by mixing with 50 μl ATP detection reagent. Finally the plate was incubated at room temperature for 20 min to allow luminescent signals to develop. The relative light unit (RLU) values were read on Thermo Varioskan Flash.

Western blot analysis. LoVo/ADR, Caco-2/ADR or HCT116/L cells were treated with different concentrations of CBF, then incubated for 48 h. Cells were washed three times and scraped in lysed RIPA containing protease inhibitors. The concentration of protein was measured using a BCA assay (Pierce Biotechnology, Rockford, IL, USA). Protein (50 μg) was loaded onto SDS-PAGE gels and then transferred to PVDF membranes. The membranes were blocked by incubating with 5% BSA for 1 h, and then incubated with the following primary monoclonal antibody: anti P-gp, anti-caspase-3, anti-caspase-9, anti-Bcl 2, anti-Bax (1:1000) overnight at 4˚C. The membrane was washed 3x15 min with TBST buffer and subsequently incubated with the HRP-conjugated secondary antibody (1:5000) for 2 h. Finally, the membranes were visualized using enhanced chemiluminescence detection (GE Healthcare Lifesciences, Pittsburgh, PA, USA), as previously described (25). β-actin was used as a loading control.

Nude mouse xenograft model. Six to eight weeks old athymic nude mice (BALB/c-nu/nu), weighing 18-24 g, were purchased

Table I. Effect of cinobufagin on the sensitivity of Lovo, Lovo/ADR, Caco-2, Caco-2/ADR, HCT116 and HCT116/L cells to anticancer drugs.

	IC_{50} (μM)	
Compound	Lovo	Lovo/ADR
DOX	1.8±0.2	16.0±0.5
+ CBF (5.0 nM)	2.1±0.3 (0.9)	4.0±0.3 (4.0)[a]
+ CBF (10.0 nM)	1.9±0.3 (0.9)	2.5±0.2 (6.4)[a]
+ CBF (20.0 nM)	1.6±0.1 (1.1)	2.0±0.2 (8.0)[b]
Verapamil (20 μM)	1.7±0.2 (1.1)	1.9±0.3 (8.0)[b]
	Caco-2	Caco-2/ADR
DOX	1.1±0.4	3.5±0.2
+ CBF (2.5 nM)	1.3±0.2 (0.8)	2.3±0.2 (1.5)
+ CBF (5.0 nM)	1.5±0.4 (0.7)	2.6±0.3 (1.4)
+ CBF (10.0 nM)	1.2±0.3 (0.9)	1.7±0.2 (2.1)[a]
Verapamil (20 μM)	1.0±0.2 (1.1)	0.9±0.1 (3.5)[a]
	HCT116	HCT116/L
L-OHP	48.8±5.0	107.3±12.1
+CBF (5.0 nM)	43.3±4.9 (1.1)	93.2±8.2 (1.1)
+CBF (10.0 nM)	41.7±6.1 (1.2)	43.5±7.4 (2.5)[a]
+CBF (20.0 nM)	38.3±3.6 (1.3)	23.3±5.0 (4.6)[a]
Verapamil (20 μM)	31.2±3.2 (1.5)	44.5±3.8 (2.4)[a]

IC_{50} values are represented as mean ± SD of three independent experiments performed in triplicate. The fold-reversal of MDR (values given in parenthesis) was calculated by dividing the IC_{50} values of substrates in the presence or absence of inhibitor by the IC_{50} of parental cells without inhibitor. [a]p<0.01 versus control group. [b]p<0.001 versus control group. HCT116/L, HCT116/L-OHP.

Table II. Effect of cinobufagin on the sensitivity of Lovo, Lovo/ADR, Caco-2, Caco-2/ADR, HCT116 and HCT116/L cells to MIT/CDF.

	IC_{50} (μM)	
Compound	Lovo	Lovo/ADR
MIT (μM)	1.05±0.09	1.23±0.08
+ CBF (20 nM)	1.08±0.06	1.12±0.07
CDF (mM)	1.02±0.11	1.13±0.05
+ CBF (20 nM)	0.95±0.10	1.01±0.08
	Caco-2	Caco-2/ADR
MIT (μM)	0.58±0.08	0.70±0.10
+ CBF (20 nM)	0.52±0.09	0.59±0.05
CDF (mM)	0.61±0.08	0.78±0.04
+ CBF (20 nM)	0.56±0.10	0.68±0.18
	HCT116	HCT116/L
MIT (μM)	0.85±0.15	1.02±0.28
+ CBF (20 nM)	0.69±0.08	1.07±0.03
CDF (mM)	0.70±0.10	0.96±0.16
+ CBF (20 nM)	0.68±0.07	0.98±0.15

IC_{50} values are represented as mean ± SD of three independent experiments performed in triplicate. HCT116/L, HCT116/L-OHP.

from Shanghai SLAC Laboratory Animal Co., Ltd. All mice were fed with sterilized food and water. LoVo/ADR ($1x10^7$) cells were resuspended in 200 μl PBS and inoculated subcutaneously into the nude mice (26). When the tumor size reached 150-200 mm^3, the mice were randomly divided into four groups (n=6 per group): saline solution (control); DOX (0.1 mg/kg); CBF (0.2 mg/kg); and DOX (0.1 mg/kg) plus CBF (0.2 mg/kg). All the drugs were administered via i.p. injection every 3 days for a total of 5 doses. The body weight of the animals was recorded every 3 days and tumor volumes (V) were calculated by the formula: V = (tumor length x $width^2$)/2.

The curve of tumor growth was drawn according to tumor volume and time of implantation. At the end of experiments, mice were sacrificed, and whole blood, tumor and other tissues (heart, liver, lung, spleen, kidney and intestine) were harvested and used for further analysis. Tumor tissues were analyzed by immunohistochemical staining (IHC) for TUNEL, Ki67 and P-gp. All of the experiments were carried out under the approval of the Administrative Panel on Laboratory Animal Care of the Putuo District Center Hospital.

Toxicity analysis. Normal tissues (heart, liver, lung, spleen, kidney and intestine) were harvested for H&E histology studies. Venous blood samples were collected in EDTA-coated tubes for hematology studies. Samples were analyzed for white blood cells (WBC), red blood cells (RBC), platelets (PLT), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and blood urea nitrogen (BUN) in the clinical laboratory at our hospital.

Statistics. Values were expressed as the mean ± standard deviation (SD). The differences between two groups were analyzed by the unpaired Student's t-test. Statistical analysis was performed using Prism 5.0. p<0.05 was considered statistically significant.

Results

Characterization of colorectal parental cells and drug-resistant cells. P-gp/MDR1 is a common biomarker of MDR. To confirm this in our cell lines, we determined the protein expression of P-gp on cell extracts with western blot analysis. As shown in Fig. 1B, LoVo/ADR, Caco-2/ADR, HCT116/L overexpressed P-gp transporter therefore showing a band at 170 kDa, whereas there parental cells have no band at the same position.

CBF sensitized P-gp-overexpressing cells to chemotherapeutic drugs. We firstly tested the cytotoxicity of CBF in different colon cancer cell lines by CCK-8 assay. The IC_{50} values were 150.8±6.9, 166.6±10.5, 145.4±8.8, 160.2±12.0, 77.5±6.6, and 79.0±4.3 nM for LoVo, LoVo/ADR, HCT116, HCT116/L, Caco-2, and Caco-2/ADR, respectively. More than

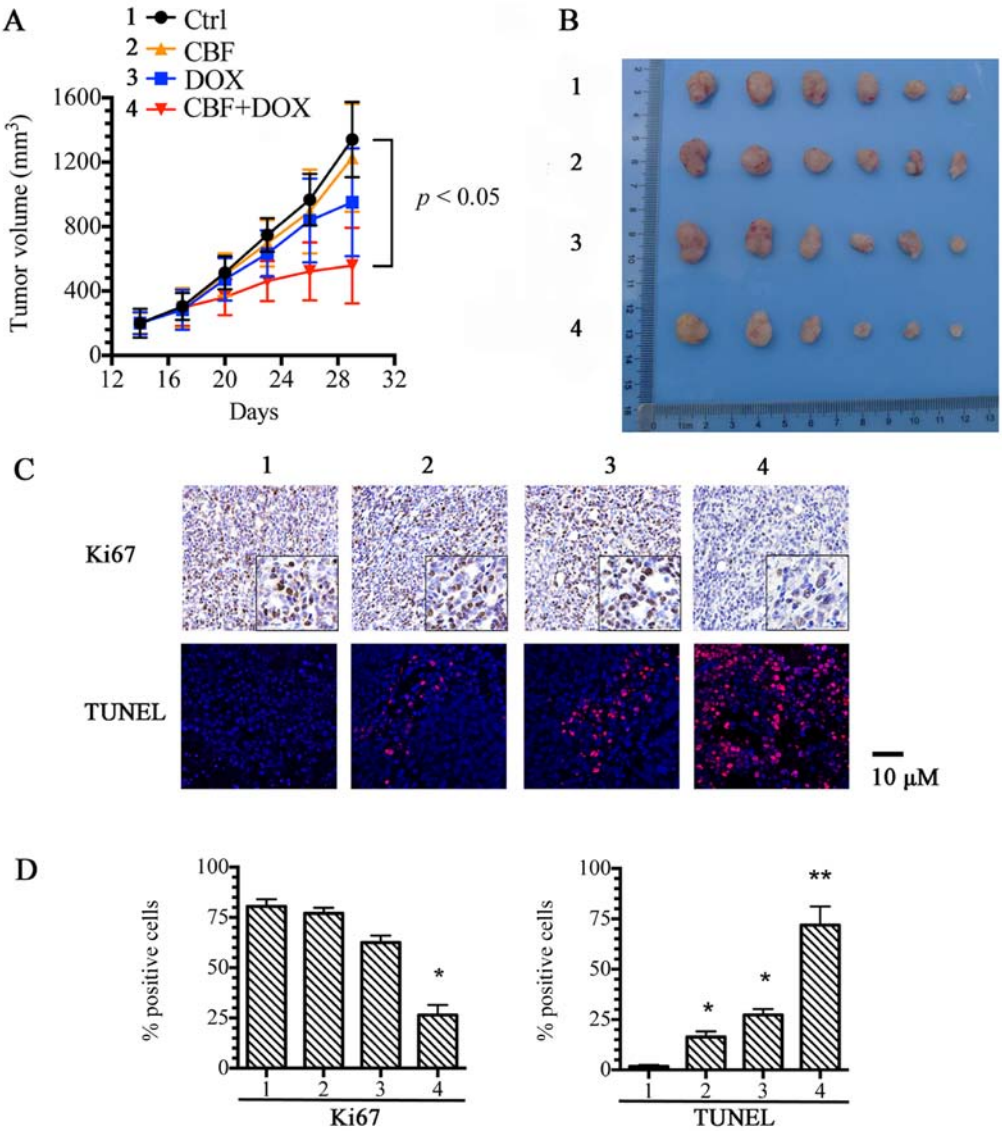

Figure 2. Potentiation of the antitumor effects of doxorubicin by cinobufagin (CBF) in a nude mouse xenograft model. (A) Changes in tumor volume with time after tumor cell inoculation. Points, mean tumor volume for each group of six mice after implantation; bars, SD. (B) Tumor size. The image was taken on the 29th day after implantation. The treatments were: control; CBF (0.2 mg/kg, i.p., q3d x5); DOX (0.1 mg/kg, i.p., q3d x5) and DOX (0.1 mg/kg, i.p., q3d x5) plus CBF (0.2 mg/kg, i.p., q3d x5, given 1 h before DOX administration). (C) IHC for Ki67 and immunofluorescence for TUNEL assay were performed in tumor tissues at the end of experiments. (D) The positive rate of Ki67 and TUNEL are based on IHC. Scale bar represents 10 μm. *p<0.05, comparing with control group, **p<0.01, comparing with control group.

85% of the cells survived at 20 nM CBF in LoVo, LoVo/ADR, HCT116, and HCT116/L cells, and at 10 nM in Caco-2 and Caco-2/ADR cells (Fig. 1C). Based on these data, CBF concentrations of 20 nM (LoVo, LoVo/ADR, HCT116, and HCT116/L) or 10 nM (Caco-2 and Caco-2/ADR) were chosen as the maximal safe concentrations for the reversal assays. The IC_{50} values of the anticancer drugs (DOX or L-OHP) in sensitive and resistant cells, with or without different concentrations of CBF, are shown in Table I. CBF decreased the IC_{50} values of L-OHP in HCT116/L cells, as well as the IC_{50} values of DOX in LoVo/ADR and Caco-2/ADR cells, but no effect was observed on their parental cells (Table I). The fold-reversal (RF) of CBF to DOX was 8.0 and 2.1 at the given concentration of CBF in LoVo/ADR and Caco-2/ADR, respectively. Similar to DOX, the RF value of CBF to L-OHP was 4.6 at 20.0 μM CBF in HCT116/L, which was superior to that of 20 μM verapamil. However, we found that CBF has no effect on MIT (mitoxantrone, BCRP substance) or CDF [5(6)-carboxy-2',7'-dichlorofluorescein, MRP2 substance] (Table II). These findings suggested that CBF significantly improve the efficacy of P-gp substrate drugs in resistance cells, indicating that CBF may be a potent reversal agent of P-gp-mediated MDR *in vitro*.

CBF reverses P-gp mediated MDR in nude mouse xenograft model. In order to substantiate our observation, we established an *in vivo* LoVo/ADR cell xenograft model in BALB/c-nu/nu mice to investigate the efficacy of CBF to reverse resistance to DOX. There was no apparent difference in tumor size between mice treated with saline, DOX, or CBF alone. However, the combination of CBF and DOX produced a significant decrease in tumor size compared with the other three groups, with an inhibition rate of 40.9% (Fig. 2A and B). As shown in Fig. 2C and D, the results showed that the cell proliferation of the

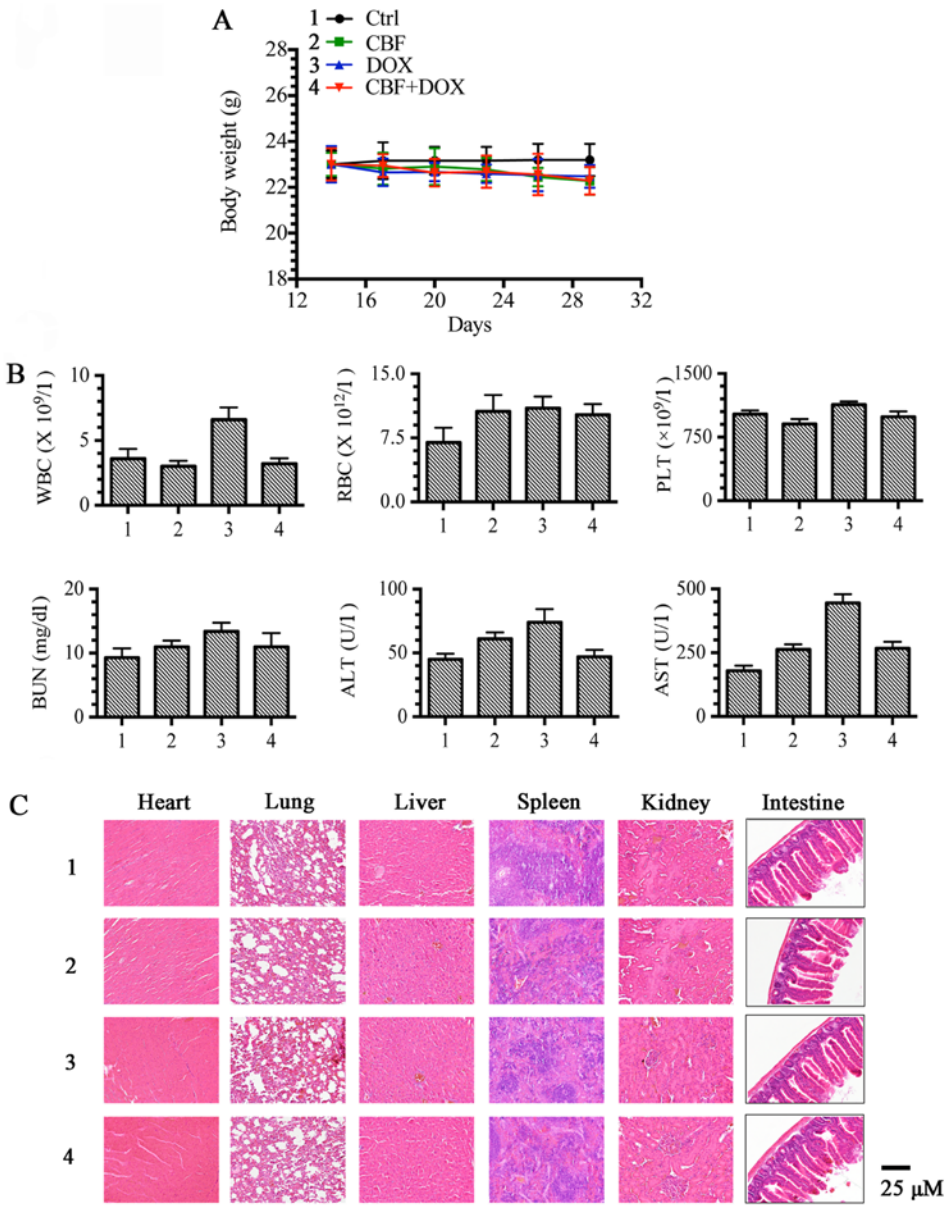

Figure 3. Toxicity analysis. (A) Changes in the weight of mice with time after tumor cell inoculation. Points, mean mouse weight for each group after implantation; bars, SD. (B) Blood analysis of mice after treatment with CBF, DOX or DOX+CBF. Error bars represent ± SD. (C) H&E histology of various organs after treatment of Nu/Nu mice bearing Lovo/ADR cancer xenografts with DOX, CBF or in combination (n=6 mice per group). Scale bar, 25 μm.

combined group was decreased compare with the other three groups (Ki67 level), and the apoptosis rate was increased (TUNEL assay). This result indicated that CBF could increase the anticancer activity of DOX *in vivo*.

Combination of CBF and DOX had no significant toxicity in vivo. Furthermore, we investigated the toxicity effect of CBF and DOX *in vivo*. As shown in Fig. 3A, none of the test subjects lost body weight or died in any of the four groups at the doses tested. Moreover, the routine blood parameters tested (WBCs, RBCs, PLTs), liver (ALT and AST) and kidney (BUN) function were in the normal range, without significant changes (Fig. 3B). Histopathology of harvested tissues (heart, liver, lung, spleen, kidney and intestine) displayed no abnormal changes as compared with normal tissues after treatment with DOX, CBF, or the combination of DOX and CBF, at the indicated doses (Fig. 3C). These findings suggest that the combination of DOX and CBF did not display any significant toxicity compared to the controls.

CBF enhances apoptosis rate of chemotherapy agents in P-gp-overexpressing cells. To determine whether cell apoptosis contributes to CBF-induced cell growth inhibition, we investigated the effects of CBF on cell apoptosis of DOX or LOHP in P-gp-overexpressing cells by flow cytometry using the Annexin V/PI staining. The results indicated that, the combination of CBF (20 nM) and DOX/LOHP greatly enhanced apoptosis of LoVo/ADR, HCT116/L, and Caco-2/ADR cells (Fig. 4A and B). Furthermore, we examined the expression of apoptosis-related proteins, such as cleaved caspase-3, caspase-9, Bcl-2 and Bax in P-gp-overexpressing cells by western blot assays. As shown in Fig. 4C, expression levels of cleaved caspase-3, caspase-9 and Bax were increased in treatment with CBF plus DOX, while levels

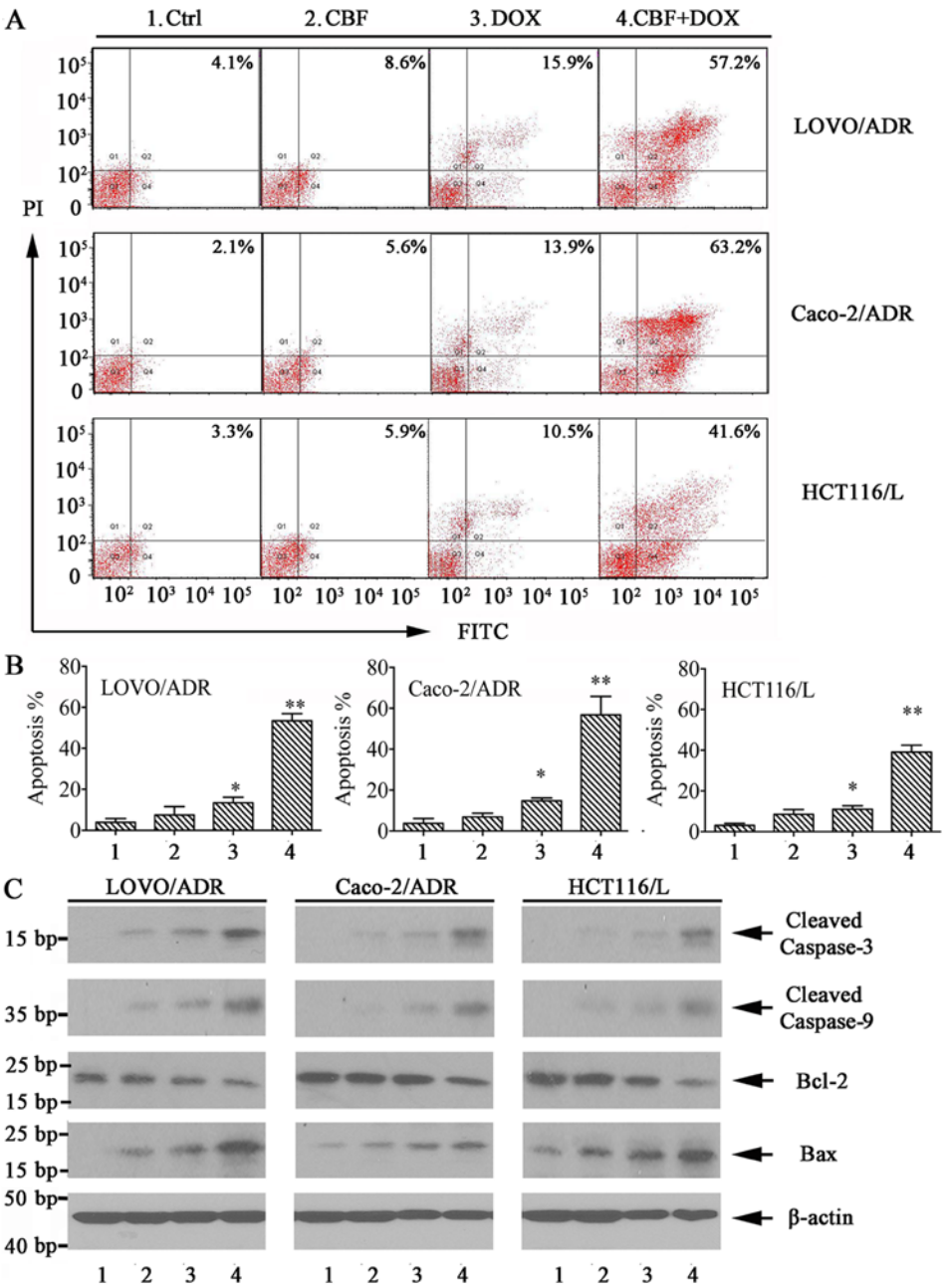

Figure 4. The effects of cinobufagin (CBF) on apoptosis of chemotherapy agents in P-gp-overexpressing cells. (A) Cell apoptosis was determined by flow cytometry with Annexin V/PI staining. Lovo/ADR cells were cultured with DOX (2 μM), CBF (20 nM) and CBF (20 nM)+DOX (2 μM) for 48 h. Caco-2/ADR cells were cultured with DOX (2 μM), CBF (10 nM), CBF (10 nM)+DOX (2 μM) and HCT116/L were cultured with LOHP (20 μM), CBF (10 nM), CBF (10 nM)+LOHP (20 μM) for 48 h. (B) Apoptosis rates are presented as the mean ± SD of the three independent experiments. (C) Western blot analysis of cleaved caspase-3, caspase-9, Bcl-2 and Bax. Effect of CBF on the protein expression of apoptosis-related proteins in MDR cells. Error bars represent the SD. Experiments were performed at least three independent times. *p<0.05 versus control group, **p<0.01 versus control group. HCT116/L, HCT116/L-OHP.

of Bcl-2 expression were reduced. These findings suggested that CBF-induced apoptosis may be involved in the enhanced cell growth inhibition of chemotherapy agents in P-gp over-expressing cells.

CBF increases the intracellular accumulation of Dox and Rho123 in P-gp-overexpressing cells. Rho123 is a P-gp substrate routinely used to study the MDR phenomenon (27). To elucidate whether the reversal ability of CBF was associated with increasing the intracellular concentration of drugs, we examined the accumulation of DOX and Rho123 in LoVo/ADR, HCT116/L, and Caco-2/ADR cells treated with CBF by flow cytometric analysis. As shown in Fig. 5, intracellular fluorescence intensity (MFI) of DOX and Rho123 was increased compared with control in the presence of CBF in MDR cells. These findings showed that CBF increased the intracellular concentration of DOX and Rho123 in a dose-dependent manner, indicating that CBF is able to inhibit P-gp-mediated drug efflux, thus enhancing the intracellular accumulation of chemotherapeutic drugs.

CBF inhibited P-gp-mediated drug transport. The permeability coefficients (Papp) of DOX (20 μM) were tested in a Caco-2 cell monolayer model (Table III). The absorbable permeability

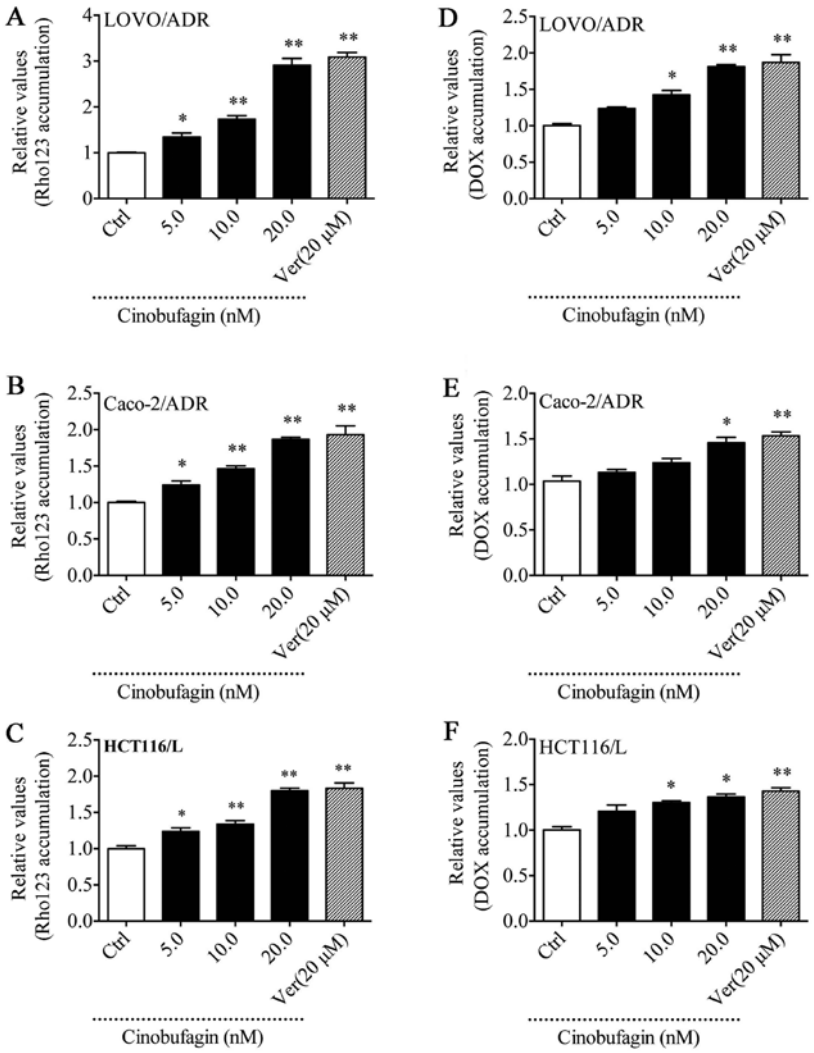

Figure 5. Effect of cinobufagin on the intracellular concentration of DOX and Rho123. The relative values of MFI in the absence or presence of cinobufagin or verapamil (positive control) at 20 μM was measured by flow cytometry in Lovo/ADR in Caco-2/ADR and in HCT116/L cells. The results are presented as fold change in fluorescence intensity relative to control MDR cells. Error bars represent the SD. Experiments were performed at least three independent times. *p<0.05 versus control group, **p<0.01 versus control group. HCT116/L, HCT116/L-OHP.

Table III. Papp and ER values of Dox in the absence or presence of cinobufagin.

Compound	Papp ($x10^{-6}$)		ER
	A to B	B to A	
DOX	0.725±0.10	3.65±0.52	5.03
DOX + CBF (20 nM)	1.154±0.12^{b}	3.03±0.40^{a}	2.63^{c}
DOX+Verapamil (20 μM)	1.295±0.03^{b}	2.76±0.28^{b}	2.13^{c}

Data are presented as mean values ± SD (n=3). ^{a}p<0.05, comparing with Dox group. ^{b}p<0.01, comparing with Dox group. ^{c}p<0.001, comparing with Dox group.

coefficient [Papp(A-to-B)] of DOX determined from the apical (A) to the basolateral (B) side was 0.725±0.10x10^{-6} cm/sec and the secretory permeability coefficient [Papp(B-to-A)] of DOX obtained from the B to the A side was 3.65±0.52x10^{-6} cm/sec. The efflux ratio (ER), which is the ratio of Papp(B-to-A)/Papp(A-to-B) was determined for assessing drug transport across the cell membrane. The presence of CBF at 20 nM reduced the ER values (2.63) compared with untreated control monolayers (5.03), demonstrating that CBF was able to significantly circumvent P-gp-mediated transport of DOX in the monolayers.

CBF inhibits P-gp ATPase activity but had no effect on P-gp expression. In order to evaluate the effect of CBF on the P-gp ATPase activity, we determined P-gp-mediated ATP hydrolysis at different concentrations of CBF. In this assay the residual ATP level, which is inversely correlated to the activity of P-gp ATPase, was measured as luminescence in relative light units (RLU). The results demonstrated that CBF inhibited basal- and verapamil-stimulated ATPase activity in a dose-dependent manner (Fig. 6A and B), suggesting that the inhibition mechanism of P-gp ATPase by CBF was non-competitive.

To further test whether the reversal ability of CBF was mediated by affecting the expression of P-gp, the effects of

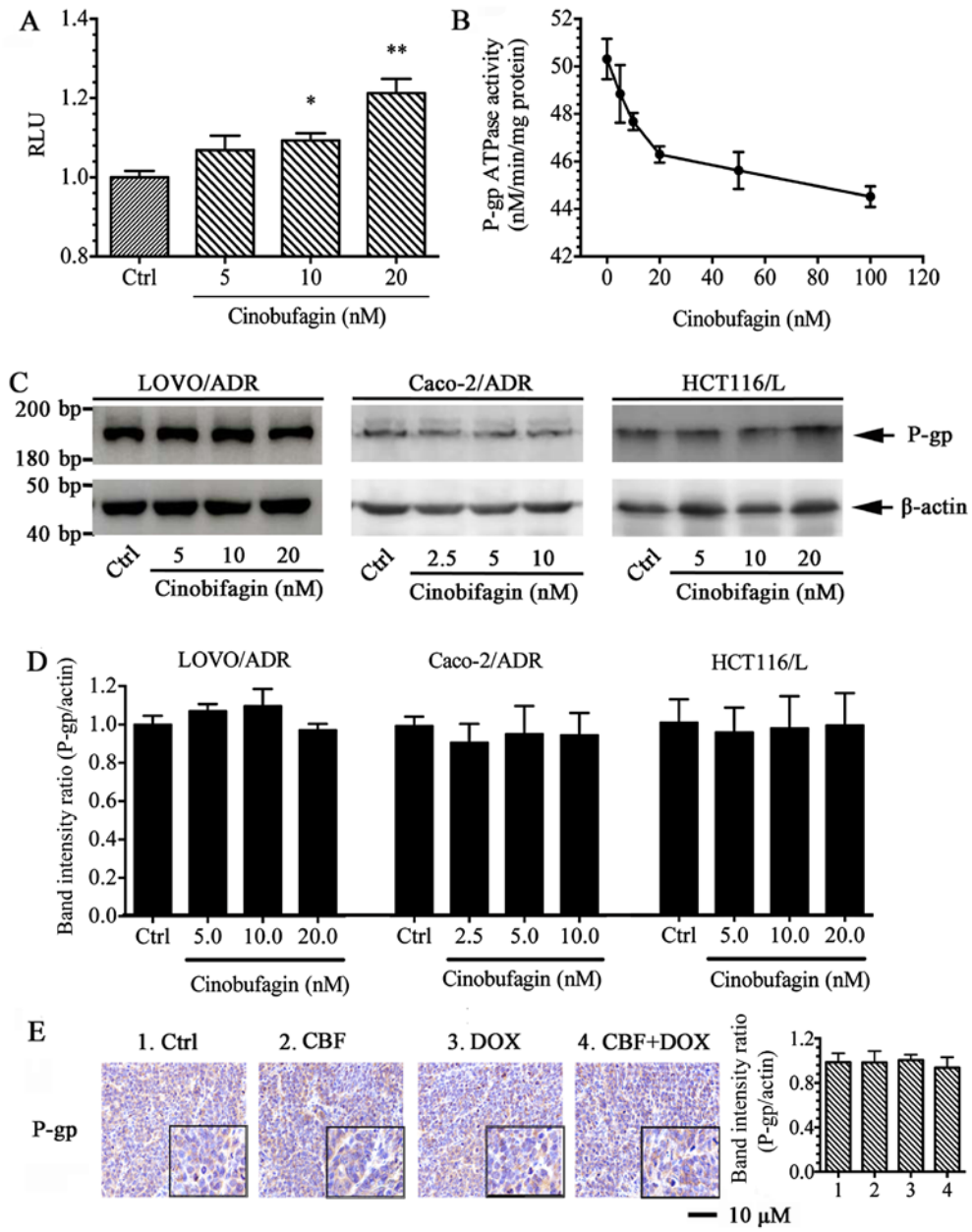

Figure 6. Effect of cinobufagin on P-gp ATPase activity and the protein expression of P-gp. (A) Inhibitory effect on RLU values by cinobufagin. (B) Inhibitory effect on verapamil stimulated ATPase activity by cinobufagin. (C) Western blot analysis of P-gp. Effect of cinobufagin on the protein expression of P-gp in MDR cells. (D) Quantitative analysis of effects of cinobufagin on P-gp expression. (E) The expression of P-gp in tumor tissues was assessed by IHC staining. *p<0.05, comparing with control group; **p<0.01, comparing with control group.

CBF on P-gp expression in MDR cells were analyzed by western blotting (WB). Results indicated that, treatment with CBF at different concentration did not alter the ABCB1 expression compared to the negative control in LoVo/ADR, HCT116/L and Caco-2/ADR cells (Fig. 6C and D). The immunohistochemical analysis further supported the results that the P-gp protein level remained unchanged *in vivo* (Fig. 6E). These results indicated that the circumvention of P-gp-mediated MDR by CBF resulted from the inhibition of P-gp transporter function but not the expression level of P-gp.

Discussion

Multidrug resistance is the main reason for the failure of cancer chemotherapy. Overexpression of ABC transporters is the most important mechanism of multidrug resistance (28). P-gp is the most studied ABC transporter, which can expel a wide range of anticancer agents from cancer cells, resulting in drug resistance. Currently, very few P-gp inhibitors are in clinical development, and the adverse drug reactions seen during the clinical trials of all three generations of P-gp inhibitors to date have prevented them continuing past the clinical trial stage (29). Therefore, developing novel reversal agents is a crucial and urgent goal for overcoming multidrug resistance. Discovering effective active ingredients or prodrugs that reverse MDR from traditional Chinese medicines, and combining them with cytotoxic drugs has been a promising strategy to overcome tumor multidrug resistance (30-32). CBF is an effective active ingredient of ChanSu, and has been proven to have antitumor activity. The proposed mechanisms

of action have been associated with killing tumor cells, inhibiting multiplication, inducing differentiation, inducing apoptosis, and anti-angiogenesis. However, the effect of CBF on MDR remains unknown. Our investigation, for the first time, assessed the potential activity of CBF in overcoming P-gp-mediated multidrug resistance both *in vivo* and *in vitro*.

During *in vitro* experiments CBF demonstrated a strong reversal effect of MDR in P-gp-overexpressing LoVo/ADR, HCT116/L and Caco-2/ADR cells, and effectively restored the sensitivity of DOX and L-OHP in drug resistant cells. However, we found that CBF has no effect on MIT or CDF, which are not P-gp substances (33,34). These results suggested that the reversal ability of CBF was specific to inhibit P-gp. Further evaluation included study of apoptosis rate of chemotherapeutic drugs and Rho123 accumulation in drug-resistant cells using flow cytometry. It was demonstrated that CBF can greatly enhance apoptosis of DOX/LOHP and accumulation of DOX and Rho123 in a concentration-dependent fashion in drug resistant cells. These data were in accordance with that of the CCK-8 assays, together demonstrating that CBF sensitized P-gp overexpressing cells to chemotherapy drugs by enhancing cell apoptosis and increasing their intracellular concentration.

The reversal effect of CBF can be due to inhibiting expression of P-gp or affecting its transport function (35-37). To fully explore the potential mechanisms, P-gp protein expression and P-gp ATPase activity were further studied. We studied the influence of CBF on P-gp expression in drug-resistant cells using the western blot method. Our results show that CBF does not alter P-gp protein expression at the concentrations used, which demonstrated reversal of MDR. Therefore, we suspect that the reversal effect of CBF is due to inhibiting the transport function of P-gp. Since ABC transporters utilize the energy released from ATP hydrolysis to transport different substrates across the cell membrane, testing ATPase activity is another extensively used method to assess the inhibition of ABC transporters (38). It was demonstrated that CBF inhibits the activity of the P-gp ATPase in a concentration-dependent manner. Based on their effects on ATPase activity, transporter modulators could be catagorized into three distinct classes. The 1st class reduced its ATPase activity at high dose but enhanced its activity at low dose. The 2nd class increased the ATPase activity in a concentration-dependent fashion. The 3rd class inhibited both basal- and verapamil-stimulated ATPase activity (39-42). CBF should be classified under the third class of modulator and belongs to non-competitive inhibitor group. As reported, ABC transporters are large, membrane-bound proteins consisting of two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs) which mediate the active transport of substrate out of the cell (43). An ATP-modulator could either bind to the drug-binding pocket in TMD as a competitive inhibitor, or block ATP-binding in NBDs as a non-competitive inhibitor, which do not compete for active sites with the normal substrate, but change the molecular structure of active sites, making it unsuitable for the inhibitors (44). Furthermore, how CBF binds to NBDs warrants further investigation.

In vivo, we observed the inhibitory effect of CBF in the LoVo/ADR nude mouse xenograft model. We found that the combination of DOX and CBF significantly increased the efficacy of the antitumor activity of DOX, without inducing any significant toxicity *in vivo*. Moreover, the P-gp protein level remained unaltered as demonstrated by IHC and WB, both *in vitro* and *in vivo*.

Most of the reported P-gp inhibitors, such as verapamil, can circumvent MDR by inhibiting the protein expression of P-gp. Nevertheless, normal expression of P-gp is an important normal physiological defense mechanism, since P-gp inhibits absorption of toxins through the small intestine, facilitates excretion of drugs and other metabolites from the liver, prevents xenobiotics from passing through blood-brain barrier (45,46). Therefore, a better way to reverse P-gp mediated multidrug resistance is to inhibit its transport function rather than affect its expression. Therefore, CBF has been demonstrated to be a safe and effective P-gp reversal agent worthy of further research.

In conclusion, this study demonstrated that CBF reverses P-gp-mediated MDR by inhibiting the efflux function of P-gp via non-competitive inhibition of P-gp ATPase activity. The efficacy and relative safety of using CBF in combination with DOX *in vivo* was demonstrated using the nude mouse xenograft model. These results indicate that CBF may have the potential to be used as an adjuvant therapy in combination with current chemotherapies to augment cancer chemotherapy and prevent or mitigate MDR.

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