

Cardamonin Enhances Cisplatin Chemosensitivity of Nasopharyngeal Cancer Cells via Attenuating c-Myc-Mediated β -Catenin/ABCG2 Signaling

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Research

Keywords: Cardamonin, ATP-binding cassette transporters, cisplatin, multi-drug resistance, nasopharyngeal carcinoma, β -catenin/ABCG2 signaling

Posted Date: June 30th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-38127/v1>

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Abstract

Purpose: Resistance to chemotherapeutic drugs in nasopharyngeal carcinoma (NPC) remains a major obstacle of clinical therapy. To address the issue, screening for natural low-toxicity products as chemosensitizers has become a promising strategy for cancer therapy. In this study, we investigated chemosensitizing effects of cardamonin (CM), a plant-derived chalcone, on cisplatin (DPP)-resistant NPC cells, and explored the molecular mechanism for its antitumor activity.

Methods: The chemotherapeutic efficacy of cardamonin, cisplatin and their combination in cisplatin-resistant NPC cells were analyzed using MTT assay, apoptosis assay, and cell cycle analysis. Real-time PCR, western blotting, and cell transfection analysis were performed to assess the synergistic inhibitory action of cardamonin supplemented with cisplatin on Wnt/ β -catenin/ABCG2 signaling. The effect of cardamonin on ABCG2 drug efflux function was analyzed by doxorubicin accumulation assay. A CNE2/DPP nude mouse model was used to determine the combinatorial effects of cardamonin on tumor growth in vivo.

Results: Cardamonin increased cisplatin-induced cytotoxicity, accompanied by induction of apoptosis and cell cycle arrest in DPP-resistant NPC cells. Moreover, cardamonin could synergize with cisplatin to downregulate β -catenin, c-Myc, and ABCG2. Specifically, cardamonin inhibited Wnt/ β -catenin/ABCG2 signaling through c-Myc-mediated transcription inactivation, thereby suppressing the expression of ABCG2 in cisplatin-resistant NPC cells. These findings were confirmed in vivo, wherein cardamonin treatment with cisplatin resulted in reduced tumor growth in a CNE2/DPP xenograft animal model.

Conclusions: Taken together, our data firstly demonstrated that cardamonin increased chemosensitivity of nasopharyngeal cancer cells to cisplatin through inactivation of Wnt/ β -catenin/ABCG2 signaling, more specifically by inhibition of β -catenin/ABCG2 signaling through c-Myc-mediated transcriptional inactivation, thereby downregulation of ABCG2 and reversal of cisplatin resistance. Thus, in addition to its chemotherapeutic potential, cardamonin may serve as a useful chemosensitizer to conventional chemotherapeutic drugs in the treatment of nasopharyngeal carcinoma.

Introduction

Nasopharyngeal carcinoma (NPC) or nasopharynx cancer, is the most common head and neck tumor that is prevalent in southern China and Southeastern Asia [1, 2]. Although NPC is generally a good response to radiation therapy, cisplatin-based combinational chemoradiotherapy is required for locally advanced cancers [3, 4, 5] and the 5-year overall survival rate still remains low [6]. One serious challenge with

cisplatin-based chemotherapy is the acquisition of chemoresistance due to increased expressions of ATP-binding cassette (ABC) transporters that is a key mechanism that contributes to multidrug resistance [7, 8, 9]. Consequently, there is an urgent need for developing new therapeutic agents for circumventing cisplatin resistance in the treatment of NPC.

Increasing evidence indicates that the Wnt/ β -catenin signaling is closely correlated to DPP resistance in several human tumors [10, 11, 12]. Moreover, abnormal Wnt/ β -catenin signaling activation contributes to upregulation of drug efflux ABC transporters in many cancer types [13, 14, 15, 16], including NPC [17]. ABCG2 has been reported as one of at least three human ABC transporters that facilitate efflux of conventional chemotherapeutic drugs including DPP[18]. Additionally, ABCG2 was shown to mediate DPP resistance, which was reversed by inhibiting wnt/ β -catenin signaling in ovarian cancer[16]. Therefore, a combination of chemotherapy targeting abnormal Wnt/ β -catenin/ABCG2 signaling has become a promising cancer therapy to improve tumor chemosensitivity and eliminate cancerous cells.

Recently, the use of selective, potent, and relatively non-toxic natural compounds to impede tumors and enhance chemosensitivity has gained immense importance[19]. Moreover, some natural plant products exhibit excellent in inhibiting Wnt/ β -catenin signaling and improving chemosensitivity in some cancers[20]. Thus, the natural low-toxicity compounds might be promising agents to enhance the therapeutic efficacy of conventional chemotherapeutic drugs for drug-resistant tumors. Cardamonin, a natural chalcone extracted from cardamom spice, has been reported extensively to have anti-inflammatory and anti-tumor activities[21]. However, whether CM can chemosensitize resistant NPC cells to DPP remains unexplored. In this report, we firstly demonstrated that CM could circumvent chemoresistance to DDP in resistant NPC cells by blocking β -catenin/ABCG2 signaling pathway. Furthermore, we identified major ABC transporter ABCG2 to be downregulated following co-treatment of CM and DPP

via c-Myc-mediated transcription inactivation in DPP resistant NPC cells. Finally, a xenograft mouse model was established to validate our in vitro results and further revealed that the combination of cardamom and DPP significantly reduced tumor growth more effectively than when used alone in vivo. Collectively, these data indicate that CM inhibits NPC growth and reverses the DPP resistance of NPC through inactivation of c-Myc-mediated β -catenin/ABCG2 signaling.

Materials And Methods

Reagents

Cardamonin, cisplatin, and other agents were purchased from Sigma-Aldrich Company. Primers were obtained from Thermo Fisher Scientific. Antibodies against ABCG2, β -catenin, α -tubulin, c-Myc, SOX2, and cyclin D1 were from Cell Signaling Technology. The secondary antibodies were purchased from Santa Cruz.

Cell culture

The human nasopharyngeal carcinoma cell line CNE2 and its cisplatin-resistant cell line (CNE2/DDP) selected by continuous exposure of parental CNE2 cells to increasing concentrations of DPP over a

duration of 9 months, were obtained from the Chinese Academy of Sciences (Beijing, China). Both cell lines were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum, 100U/ml penicillin, and 100ug/ml streptomycin. CNE2/DDP cells were continuously maintained in culture medium in the presence of 2mM DDP and grown in drug-free medium for two weeks before experiments.

Cell cytotoxicity assay

MTT assays as described previously [22] were conducted to evaluate the ability of CM to enhance DPP cytotoxicity in CNE2 and CNE2/DPP cells. Cells were treated for 72h with different concentrations of CM and DPP or either agent alone. The cytotoxic effects of CM and DPP were measured by a microplate reader. IC50 (inhibitory

concentration 50%) values were calculated using GraphPad Prism 7.0 (GraphPad Software Inc. San Diego, CA).

Cell apoptosis assay

Cell Apoptosis was investigated using an Annexin V-FITC/PI double staining apoptosis detection kit according to the manufacturer's instruction. 5×10^5 cells per well were seeded into six-well plates and treated with CM and DPP or either drug alone for 24h, and then the cells were collected and washed twice with PBS (0.01M, pH7.2). Apoptotic cell fraction was analyzed by flow cytometry.

Cell cycle analysis

The analysis of cell cycle was performed by a flow cytometry (BD, San Diego, CA). Cells were treated by DPP supplemented with or without CM for 24h, and then the cells were harvested, fixed in cold ethanol, and stored at -20°C overnight. The cell cycle was analyzed by flow cytometry using after the fixed cells were suspended in PI/RNase staining buffer.

Real-time PCR analysis

Total RNAs were isolated from cells treated with different conditions using the Qiagen RNeasy Mini Kit and the relative quantification of mRNA was performed using the SYBR green assay kit (Takara, Dalian, China). The expression of GAPDH was applied for normalization of the expression of each gene, and relative expressions of indicated genes were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method [23]. Primer sequences are presented in Table 1

Western blot analysis

Cells were collected after treatment for 24h under different conditions and total cell lysates using RIPA lysis buffer were obtained from the treated cells. The protein concentrations were determined using Bio-Rad protein assays (Sigma-Aldrich, Shanghai, China) and all experimental samples were normalized by α -tubulin as a reference protein. Proteins from cell lysates were separated on 12% SDS-PAGE,

following by electrotransferring to PVDF membranes. The membranes were blocked with 5% BSA and incubated with specific primary antibodies at 4°C overnight. Then it was incubated with secondary antibodies at room temperature for 1h. Finally, proteins of interest were visualized with the ECL detection system (Millipore Co.) following the manufacturer's protocol.

Cell transfection

The pcDNA3- β -catenin, pcDNA3-c-Myc, and empty plasmids (pcDNA3) were constructed by GenePharma (Shanghai, China). The small interfering RNAs (siRNAs) targeting β -catenin and c-Myc or their scramble siRNAs were purchased from Ribobio Company (Guangzhou, China). For transient transfection, overexpressing plasmids or siRNAs of indicated genes were individually transfected into CNE2/DPP cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The control groups were generated by infecting CNE2/DPP cells with empty plasmid or scramble siRNAs.

Drug accumulation assay

The effect of CM on ABCG2 activity was assessed by measuring intracellular doxorubicin(DOX) accumulation that is a conventional substrate chemotherapeutic agent of ABCG2[24]. CNE2 and CNE2/DPP(5×10^5 per well) cells were seeded into six-well plates respectively and treated by 4 μ M doxorubicin with or without various concentrations of CM for 1h. Then, the cells were collected and washed twice with ice-cold PBS and finally resuspended in 100 μ L of ice-cold PBS for DOX-associated MFI measured by flow cytometry. Fumitremorgin C (FTC, a specific inhibitor of ABCG2 that can block the pumping out of doxorubicin in overexpressing ABCG2 cells[24]) was used as the positive control.

In vivo xenograft assay

To evaluate the in vivo effects of CM on CNE2/DPP cells, a CNE2/DPP xenograft model was established. Six-week-old BALB/c nude mice were purchased from the

Medical Laboratory of the University of Zhengzhou (Zhengzhou, China). CNE2/DPP cells (5×10^6) were injected subcutaneously into the left flank of the mice. Three days after injection, the mice were randomized into four groups: Control (PBS, 0.1ml/10kg), DPP (5mg/kg), CM (25mg/kg), DPP (5 mg/kg)

and CM (25 mg/kg). Five mice were set up for each group and every group was administered twice a week by intraperitoneal injection. Tumor volume was measured at 3-day intervals with a caliper and calculated as $(\text{length} \times \text{width}^2) / 2$ for four weeks. After 4-week drug treatment individually or in combination, nude mice were humanely euthanized and xenograft tumors were harvested and measured.

Statistical analysis

All analysis was conducted using GraphPad Prism 7.0 (GraphPad Software Inc. San Diego, CA). Data are expressed as the mean \pm SD of triplicate samples. Differences were analyzed by the Student *t* test and One-way analysis of variance. P-value < 0.05 was accepted as indicative of significant differences.

Results

Cardamonin increases the chemosensitivity to DPP in DPP-resistant NPC cells

To determine the effects of CM on DPP-resistant NPC cells individually or in combination, the cytotoxicity of both drugs was analyzed using sensitive NPC cells (CNE2) and its DPP-resistant NPC cells (CNE2/DPP). The IC₅₀ values of DPP in CNE2 and CNE2/DPP cells were found to be 4.687 and 32.896 μ M respectively and Resistance Index (RI) rose to more than seven-folds, which reflected therapeutic efficacy of drug-resistant cells for DPP (Table1). Furthermore, there was markedly reduced cisplatin cytotoxicity in CNE2/DPP cells in comparison to their sensitive counterparts (Figure1A). Importantly, we found treatment with CM significantly decreased the IC₅₀s of DPP against CNE2/DPP cells in a dose-dependent manner (Figure 1C). In contrast, no significant effects were observed in CNE2 cells (Figure 1B). These findings indicate that CM potentiates chemosensitivity of DPP to CNE2/DPP cells but exerts little influence on CNE2 cells, supporting the notion that CM can attenuate chemoresistance in DPP-resistant NPC cells.

Cardamonin induces apoptosis and cell cycle arrest in DPP-resistant NPC cells

To clarify whether CM by synergizing with DPP could contribute to corresponding enhancement in apoptosis, apoptosis assays were performed following CM and DPP treatment either alone or combined. We observed that CM or DPP treatment alone significantly increased apoptosis while combined CM and DPP treatment further enhanced apoptosis in sensitive CNE2 cells (Figure 2A), indicating the increased apoptosis in the combination therapy may be primarily driven by DPP. On the contrary, in resistant CNE2/DPP cells, DPP treatment did not induce apoptosis, but the cotreatment of DPP and CM resulted in

significantly increased apoptosis (Figure 2B). These results supported that CM induced more apoptosis in resistant CNE2/DPP cells upon DPP treatment.

DPP has been reported as a G0/G1 and G2/M cell cycle arrest inducer [25]. Then, we investigated how the combined treatment with CM and DPP regulated cell cycle in both sensitive CNE2 cells and its resistant CNE2/DPP cells. Our results revealed that DPP induced G0/G1 arrest while CM induced G2/M arrest in the sensitive CNE2 cells. However, the combined CM and DPP treatment caused G0/G1 arrest (Figure 2C), suggesting that DPP regulates primarily the cell cycle dynamics in the sensitive CNE2 cells. On the contrary, in resistant CNE2/DPP cells, CM treatment caused a G2/M growth arrest, but DPP treatment had little effect on cell cycle activities. Notably, the combined CM and DPP treatment resulted in enhanced G2/M phase arrest (Figure 2D), indicating that the cell cycle dynamics is primarily driven by CM in resistant CNE2/DPP cells. These results appeared to be in line with apoptosis assays above.

Cardamonin decreases the expressions of β -catenin, c-Myc, and ABCG2 in CNE2/DPP Cells

To understand the molecular mechanisms of the therapeutic effects of the combined treatment of CM and DPP shown above, firstly, the expression levels of multidrug resistance-associated genes and Wnt/ β -catenin-associated genes were screened and analyzed by real-time PCR analysis in CNE2 and CNE2/DPP cells. We found that the resistant CNE2/DPP cells exhibited much higher expressions of β -catenin, c-Myc, and ABCG2 than the sensitive CNE2 counterparts (Figure 3A and Figure 3B). Then, the expressions of multidrug resistance-specific genes (ABCB1, MRP1, ABCG2, and CD44) and Wnt/ β -catenin-specific genes (β -catenin, Nanog, Sox2, OCT4, c-Myc, KLF4, and cyclin D1) were measured using real-time PCR analysis in CNE2/DPP cells treated with or without CM for 24h. The data showed that the transcript expressions of ABCG2, β -catenin, and c-Myc were markedly downregulated by cotreatment of CM and DPP, compared with either agent alone (Figure 3C and Figure 3D). In addition, the protein expression levels of β -catenin, c-Myc, and ABCG2 analyzed by Western Blotting in CNE2/DPP cells were also decreased in comparison to CM or DPP treatment alone (Figure 3E) after 24h of cotreatment with CM and DPP. Overall, these results suggest that CM inhibits chemoresistance through Wnt/ β -catenin/ABCG2 signaling pathway in combinational treatment.

Cardamonin attenuates c-Myc-mediated β -catenin/ABCG2 signaling

To further assess the effects of CM on the downstream target genes of Wnt/ β -catenin signaling in resistant CNE2/DPP cells, c-Myc, and ABCG2 expression were analyzed by real-time PCR following β -catenin overexpression or knockdown. We found that β -catenin overexpression upregulated c-Myc and ABCG2 gene expression, whereas β -catenin knockdown led to the opposite effect (Figure 4A and 4B).

Also, the combining CM with DPP led to a significant decline in the expression levels of the indicated genes in β -catenin knockdown CNE2/DPP cells (Figure 4B). Next, to determine whether c-Myc is critical for CM action, we set out to assess the transcript and protein expression of ABCG2 analyzed by real-time PCR and Western blotting

after c-Myc overexpression or knockdown. We found that the expression levels of ABCG2 were dramatically upregulated in c-Myc overexpressing cells (Figure 4C and 4D), whereas the opposite was observed after c-Myc knockdown in DPP-resistant cells (Figure 4E and 4F). Importantly, we observed the inhibitory effect of CM on the ABCG2 signal was weakened in c-Myc silencing cells (Figure 4E and 4F), whereas CM could at least partly counteract exogenous overexpression of c-Myc supplemented with DPP in increasing ABCG2 expression (Figure 4C and 4D), implying that c-Myc plays a crucial role in mediating the chemosensitizing effects of CM on DPP-resistant NPC cells via the downregulation of ABCG2. Taken together, these data suggest that CM enhances chemosensitivity of DPP-resistant NPC cells to DPP by attenuating c-Myc-mediated transcription activation, resulting in reduced expression of ABCG2.

Cardamonin increases accumulation of Doxorubicin

To determine whether CM could affect the activities of drug efflux transporter ABCG2, the intracellular concentration of doxorubicin (DOX) that is a conventional substrate chemotherapeutic agent of ABCG2 [26] in CM-treated CNE2 and CNE2/DPP cells were detected using doxorubicin accumulation assay. We found the intracellular accumulation of doxorubicin in the resistant CNE2/DPP cells was remarkably lower than that in the sensitive CNE2 counterparts (Figure 5). Importantly, treating with CM significantly increased the intracellular accumulation of doxorubicin in a concentration-dependent manner in CNE2/DPP, but no significant effects were observed in the sensitive CNE2 cells. In fact, the increased fluorescence in the CM-treated CNE2/DPP cells was similar to that in the FTC-treated counterparts, indicating that CM can elevate the concentration of doxorubicin inside the CNE2/DPP cells by inhibition the drug efflux function of ABCG2.

Cardamonin suppresses NPC xenograft growth

To verify the in vitro findings mentioned above, the antitumor effects of CM were evaluated using a mouse xenograft model of DPP-resistant NPC. CM could not only

reduce tumor volumes (Figure 6A) but also lower tumor weights (Figure 6B) as compared to the untreated controls. However, the combined CM and DPP treatment markedly suppressed tumor growth in comparison with CM or DPP treatment alone as shown in Figure 6C. These findings indicate that the combination of CM and DPP can inhibit tumor growth more effectively than when used alone in vivo. Also, these results were consistent with our in vitro findings.

Discussion

Chemotherapeutic drug resistance is one of the major unmet clinical challenges in NPC treatment. The natural plant products have become promising strategies in overcoming chemoresistance[20]. In this study, we for the first time demonstrate a novel molecular mechanism for CM-induced chemosensitization to DPP in DPP-resistant NPC cells by blocking the Wnt/ β -catenin/ABCG2 signaling pathway. This study reveals that the combination of CM and DPP exerted synergistic inhibitory effects on DPP-resistant NPC cell survival. Mechanismly, CM could reverse drug resistance via inhibition of β -catenin/ABCG2 signaling, which was closely correlated with suppressing c-Myc-mediated transcription in DPP-resistant NPC cells. In addition, a xenograft animal model was established to confirm the in vitro findings, indicating CM as adjunctive therapy to DPP can inhibit tumor growth of NPC and circumvent drug resistance in vitro and in vivo.

It is crucial to understand the occurrence of chemoresistance, and eventually how to prevent it for combatting cancer effectively [8, 27, 28], and it is well known that the ABC transporter family are implicated in multidrug resistance and high-expressed levels of ABCG2 have a greater capacity to expel therapeutic drugs [25,29,30]. Furthermore, accumulating findings reveal that aberrant Wnt/ β -catenin signaling and increased ABCG2 expression are closely correlated to multidrug resistance in many cancers [14, 15, 31], including NPC [18, 17]. Also, ABCG2 was reported as a downstream gene of β -catenin since its seven functional transcription factor binding

sites have been identified in the ABCG2 gene promoter [16]. Therefore, targeting the aberrant Wnt/ β -catenin has become a novel approach to improve chemosensitivity in cancer treatment. Accordingly, in the present study, after screening for the expression levels of multidrug resistance-associated genes and Wnt/ β -catenin-associated genes using real-time PCR analysis in CNE2 and CNE2/DPP cells, we found that the resistant CNE2/DPP cells exhibited much higher expressions of β -catenin, c-Myc, and ABCG2 than the sensitive CNE2 cells (Figure 3A and 3B). Importantly, the three gene expressions in CNE2/DPP cells were markedly downregulated by cotreatment of CM and DPP, compared with either agent alone (Figure 3C and 3D). In addition, we found that protein expressions of β -catenin, c-Myc, and ABCG2 decreased following CM and DPP co-treatment of resistant CNE2/DPP cells using western blotting analysis (Figure 3E). These results suggest that CM overcomes chemoresistance through inhibition of Wnt/ β -catenin/ABCG2 signaling.

c-Myc as an important regulator of stem cells may be associated with tumorigenesis by activating its downstream target genes[32, 33]; Furthermore, recent studies have shown that c-Myc may dysregulate the transcription of ABC transporter genes such as binding to the promoter of ABCG2 and increase its expression in some cancers, thereby resulting in the multidrug resistance profile[34, 35, 36]. In addition, it has been reported that c-Myc is the ultimate downstream effector of the Wnt/ β -catenin pathway [37, 38]. These results suggested that c-Myc may serve as a link connecting Wnt/ β -Catenin pathway and multidrug resistance. Herein, our data demonstrated that c-Myc expression was upregulated following β -catenin overexpression, whereas β -catenin knockdown decreased the expression levels of c-Myc in

CNE2/DPP cells (Figure 4A and 4B). To determine whether c-Myc is critical for CM action, c-Myc overexpression or knockdown was conducted by transfecting overexpressing plasmids or siRNAs targeting c-Myc to CNE2/DPP cells. As shown in Figure 4E and 4F, the inhibitory effect of CM on ABCG2 expression was weakened in c-Myc silencing cells,

whereas exogenous overexpression of c-Myc could at least partly counteract CM in inhibiting ABCG2 expression (Figure 4C and 4D), implying that c-Myc plays a crucial role in mediating the chemosensitizing effects of CM on DPP-resistant NPC cells through c-Myc-mediated transcription inactivation, thereby downregulation of ABCG2.

Inhibition of ABCG2-mediated drug efflux function is emerging as a promising strategy in circumvention of drug resistance in cancer treatment. To further probe this, drug accumulation inside CNE2 and CNE2/DPP cells was measured by flow cytometry. Our data revealed that CM blocked the efflux of doxorubicin, thereby resulting in an increase of intracellular concentration of doxorubicin in a dose-dependent manner in CNE2/DPP, but had little effect on CNE2 cells. Thus, the reversal effect of CM on resistant CNE2/DPP cells could be achieved not only by downregulating transcript and protein expression of ABCG2 (Figure 3) but also by attenuating the efflux function of ABCG2 (Figure 5). In addition, combined CM and DPP treatment significantly inhibited tumor growth in vivo (Figure 6), in line with the in vitro findings.

Conclusions

In summary, the present study for the first time demonstrated that the natural compound CM could chemosensitize the resistant NPC cells to DPP by suppressing β -catenin/ABCG2 signaling through targeting c-Myc, resulting in reversed drug resistance in vitro and in vivo.

Abbreviations

CM: cardamonin

DPP: cisplatin

NPC: nasopharyngeal carcinoma

ABC: ATP-binding cassette

DOX: doxorubicin

Declarations

Ethics approval and consent to participate

All animal experiments were carried out according to the National Institutes of Health Guide for Care and Use of Laboratory Animals and approved by the Ethics Committee of Henan Provincial People's Hospital and Zhengzhou University (Zhengzhou, China). The manuscript does not contain clinical studies or patient data.

Consent for publication

The authors have agreed to publish this research article in your journal.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

All authors declare to have no conflict of interest.

Funding

No funding

Author contributions

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Consent for publication

The authors have agreed to publish this research article in your journal.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Acknowledgments

We thank Dr. Jun Wang (Zhengzhou University) for language revision.

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Tables

Table 1: List of primer sequences used in real-time PCR

Genes	Forward	Reverse
β-catenin:	5'-GCTTTCAGTTGAGCTGACCA-3'	5'-CAAGTCCAAGATCAGCAGTCTC-3'
c-Myc:	5-GCTGCTTAGACGCTGGATTT-3',	5-CACCGAGTCGTAGTCGAGGT-3'
Nanog	5'-CATGAGTGTGGATCCAGCTTG-3'	5'-CCTGAATAAGCAGATCCATGG-3'
KLF4:	5'-GTGCCCCGACTAACCGTTG-3',	5'-GTCGTTGAACTCCTCGGTCT-3'
OCT4:	5'-CTTGCTGCAGAAGTGGGTGGA-3'	5'-CTGCAGTGTGGGTTTCGGGCA-3'
Sox2:	5'-CATCACCCACAGCAAATGACAGC-3'	5'-TTGCGTGAGTGTGGATGGGATTG-3'
Cyclin D1:	5'-CCATCCAGTGGAGTTTGTGTC-3',	5'-AGCGTATCGTAGGAGTGGGA-3'
MDR1:	5'-GACATCCCAGTGCTTCAGG-3'	5'-GCCACTGAACATTCAGTCG-3'
ABCG2:	5'-CACAAGGAAACACCAATGGCT-3'	5'-ACAGCTCCTTCAGTAAATGCCTTC-3'
MRP1:	5'-GTGATGGCGATGAAGACCAAGA-3'	5'-GCCAGCTCCCAGGCATAAAA-3'
MMP2:	5'-CCGTCGCCCATCATCAAGTT-3'	5'-CTGTCTGGGGCAGTCCAAAG-3'
MMP9:	5'-GGGACGCAGACATCGTCATC-3'	5'-TCGTCATCGTCGAAATGGGC-3'
CD44:	5'-AGACAACCACAAGGATGACTGATG-3'	5'-TCCAGTTTCCTTCATAAGCAGTGG-3'
GAPDH:	5'-GCACCGTCAAGGCTGAGAAC-3'	5'-TGGTGAAGACGCCAGTGGA-3'

Table2

Cell Line	IC50 of DPP(uM)		Resistance Index(RI)
	Parent Cell	Resistance Cell	
CNE2	4.687 ± 0.542	32.896 ± 1.042	7.032 ± 0.723

Figures

Figure 1

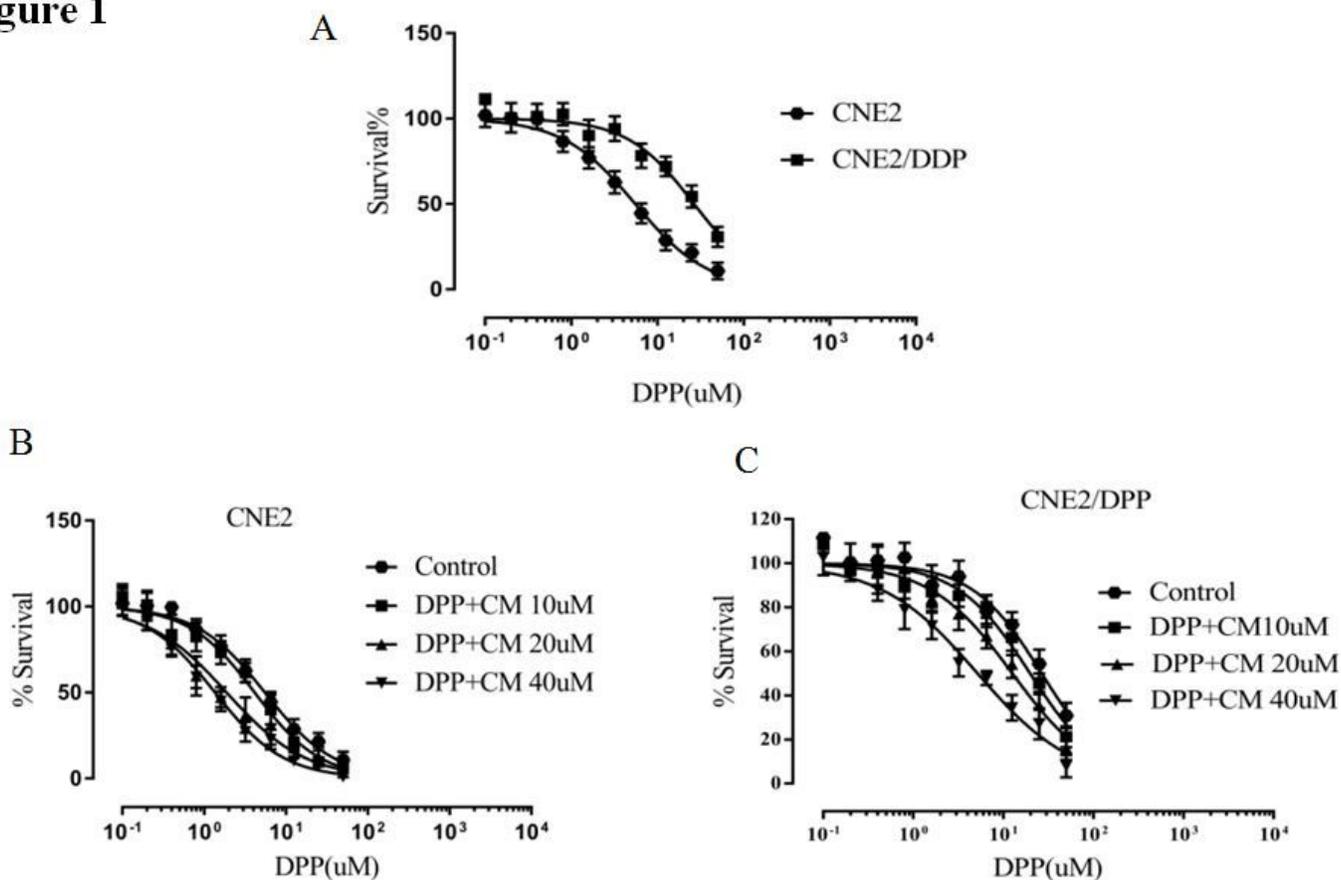


Figure 1

Cardamonin enhances DPP sensitivity in DPP-resistant NPC cells. (A) Cell activity of CNE2 and CNE2/DDP cells. (B and C) Reversal activities of cardamomin at various concentrations were determined in CNE2 and CNE2/DDP cells. (Table.2) IC50 values of DPP in CNE2 and CNE2/DDP cells. Data are presented as mean \pm SD of three independent experiments.

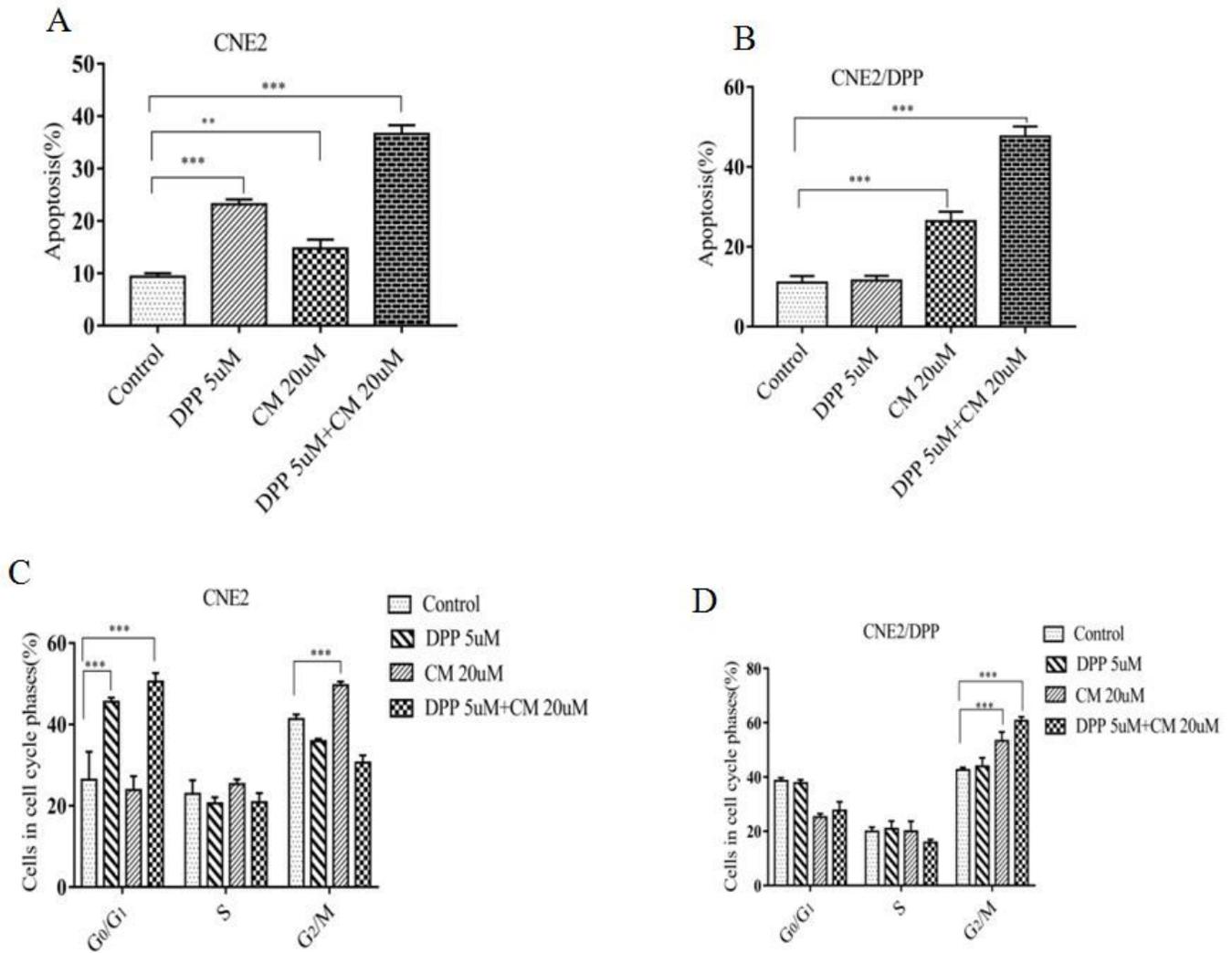


Figure 2

Cardamomin induces apoptosis and cell cycle arrest in DPP-resistant NPC cells. (A and B) Cell apoptosis detection assay for cells stained with Annexin V/PI and the apoptotic cell number was determined by flow cytometry in CNE2 and CNE2/DPP cells. (C and D) Cell cycle analysis for cells treated with cardamomin and/or DPP, followed by staining with propidium iodide and subjected to flow cytometry analysis for the determination of DNA content in CNE2 and CNE2/DPP cells. (* $P < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Similar results were obtained from three independent experiments.

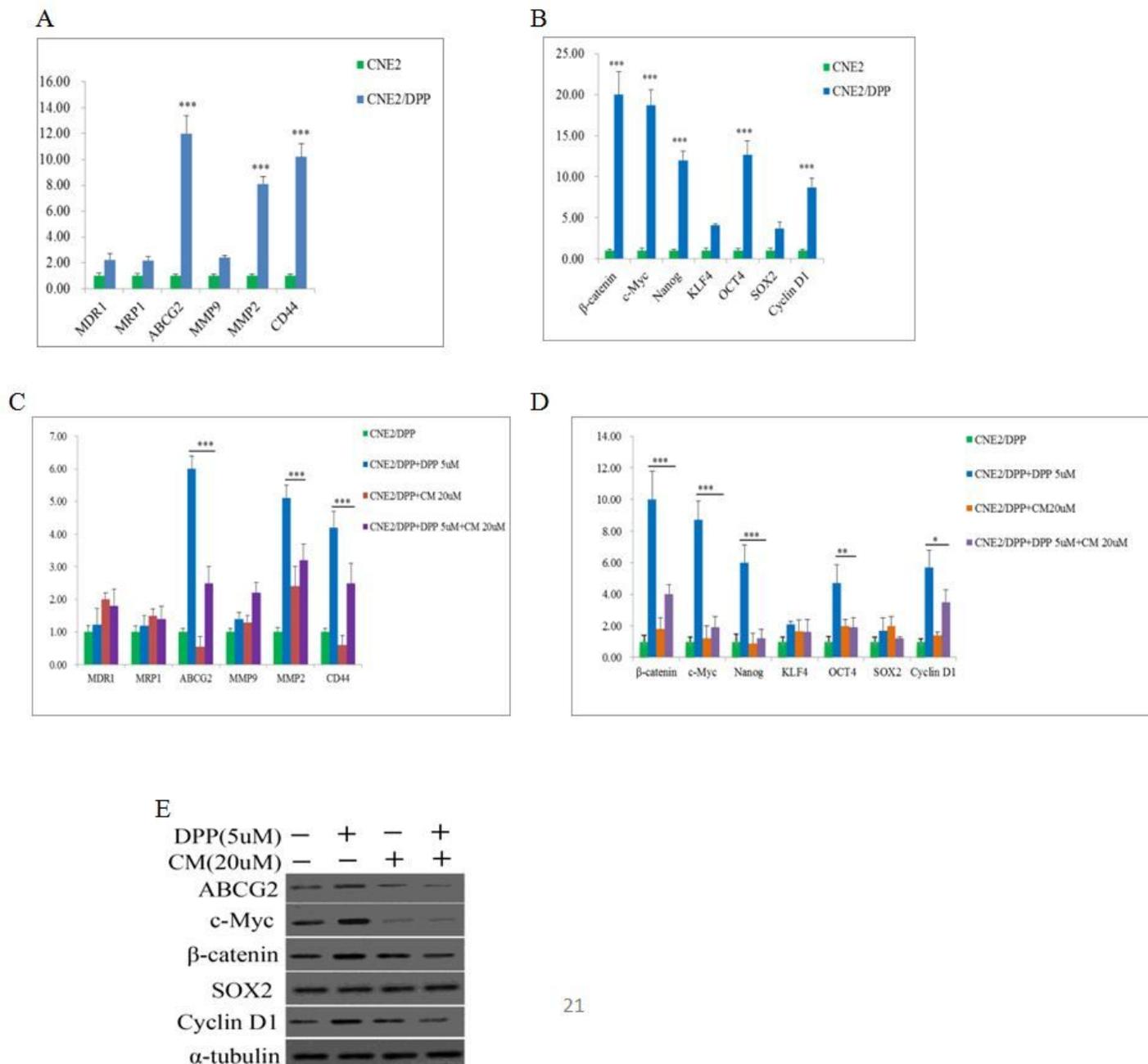


Figure 3

Cardamonin decreases the expression levels of β -catenin, c-Myc, and ABCG2 in CNE2/DPP cells (A and B). Real-time PCR analysis was performed to screen and analyse the expression levels of multidrug resistance-associated genes and Wnt/ β -catenin-associated genes in CNE2 and CNE2/DPP cells. (C and D) Real-time PCR assay was used to detect the expression levels of multidrug resistance-specific genes (MDR1, MRP1, ABCG2, CD44, MMP2, and MMP9) and Wnt/ β -catenin-specific genes (β -catenin, c-Myc, Nanog, KLF4, OCT4, SOX2, and Cyclin D1) in CNE2/DPP cells treated with or without 20uM cardamonin for 24h. Data represent the relative amounts of mRNA expression in comparison with a reference gene GAPDH, and presented as mean \pm SD (* $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$). (E) Western blot analysis

evaluating the effects of cardamomin on the protein expressions of β -catenin, c-Myc and ABCG2 in CNE2/DPP cells. The experiments were repeated three times with similar results.

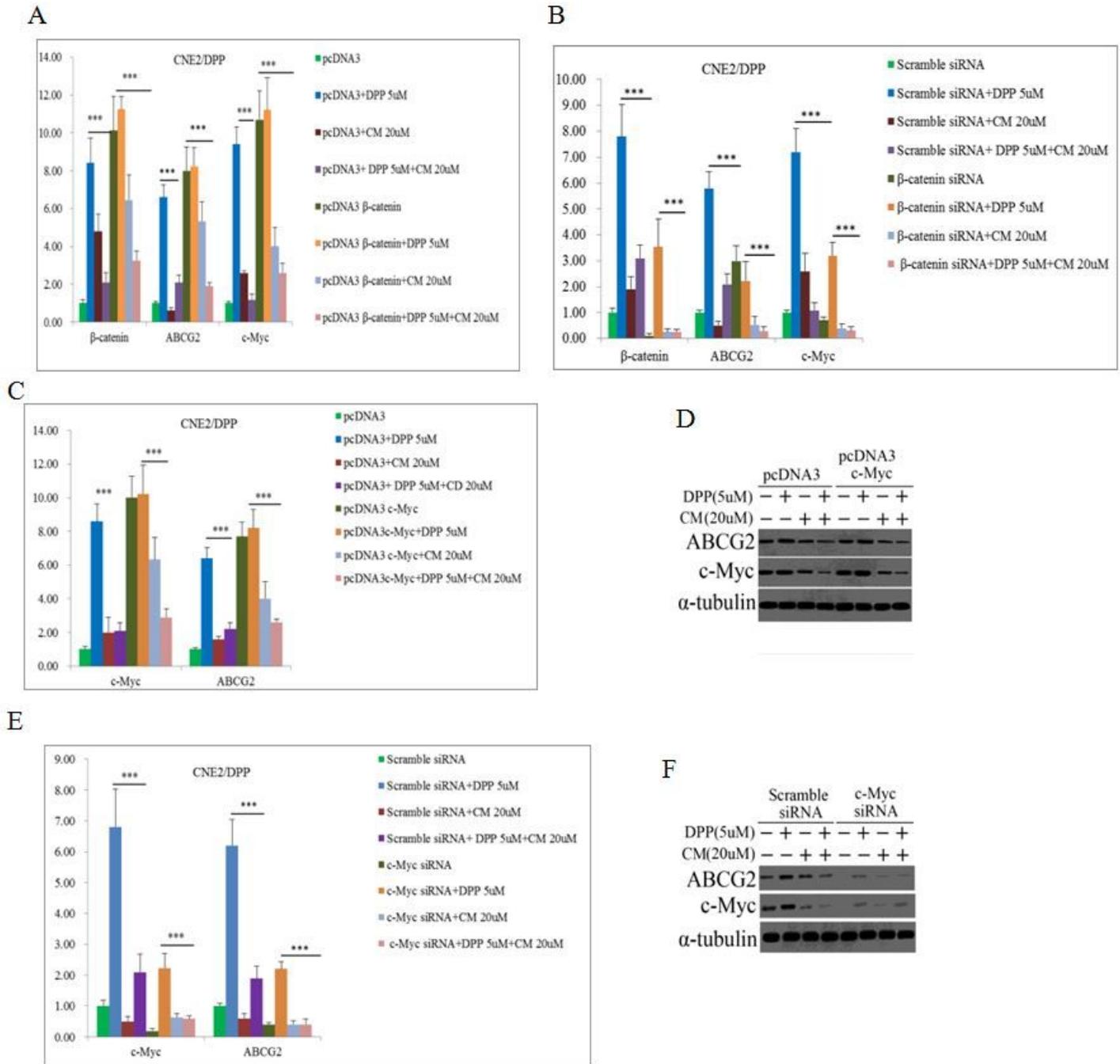


Figure 4

Cardamomin attenuates c-Myc-mediated β -catenin/ABCG2 signaling in DPP-resistant NPC cells. (A and B) Real-time PCR analysis was used to measure ABCG2 and c-Myc expression levels in CNE2/DPP cells after β -catenin overexpression or knockdown. (C and E) Real-time PCR and (D and F) Western Blotting analysis was performed to evaluate ABCG2 and c-Myc expression levels in CNE2/DPP cells following c-Myc overexpression or knockdown. All experiments were repeated at least three times (* $P < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

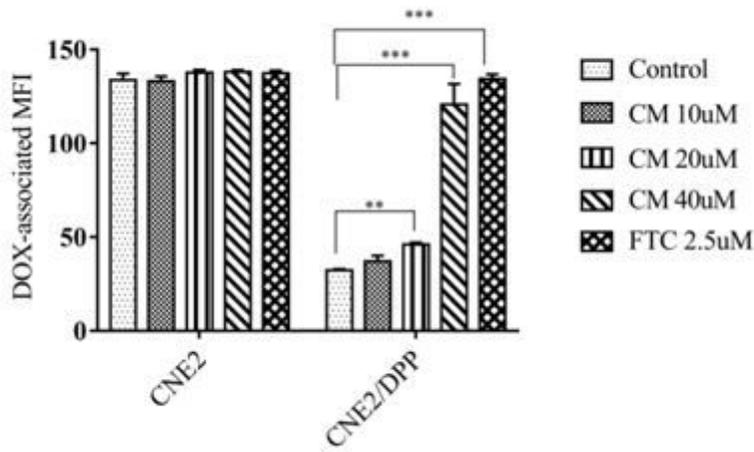


Figure 5

Cardamonin decreases the intracellular accumulation of doxorubicin in DPP-resistant NPC cells. Cells were pretreated with 0, 10, 20, and 40uM of cardamonin for 1h, and then exposed to 4uM doxorubicin for another 1h. DOX-associated MFI was measured by flow cytometry. Data are expressed as means \pm SD of three independent experiments (* P < 0.05; ** p < 0.01; *** p < 0.001 vs. control).

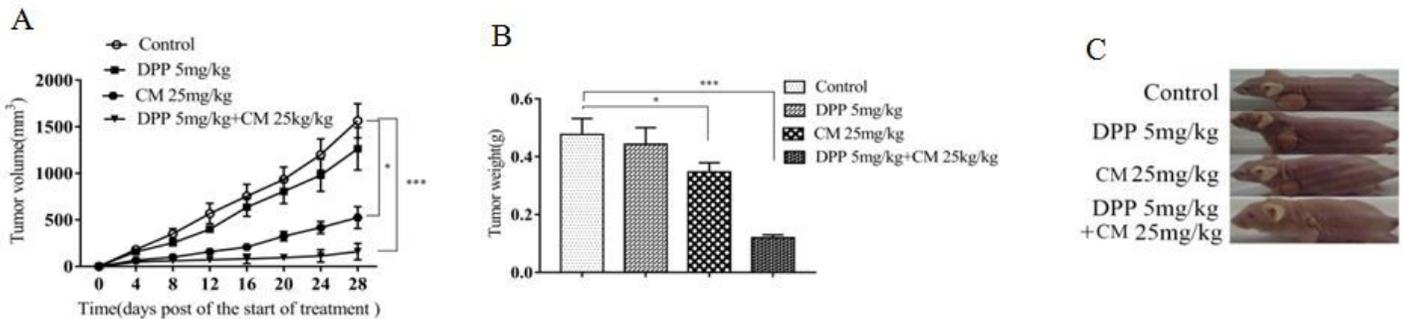


Figure 6

Cardamonin enhances DPP against DPP-resistant derived xenograft tumors. (A) The tumor weight of the mice. (B) The tumor volume of the mice. (C) Photos of tumor-bearing mice at the end of the experiments. Each point represents the mean SEM of 5 mice per group. Data represent the mean \pm SEM of three independent experiments. (n = 5 mice/group; * P < 0.05, ** P < 0.01, *** p < 0.001 vs. control).