Anti-tumour Effect of Cyanidin-3-o-glucoside Combined With Cisplatin in the Mice Xenograft Models of Cervical Cancer

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Abstract

Cervical cancer is the fourth most common carcinoma in women. Cisplatin (DDP) is the first-line drug for the treatment of cervical cancer.

Although efficacious, its application is constrained by the intolerance and serious adverse effects associated with cisplatin. Here, we aimed to investigate the *in vivo* anti-cervical cancer effects of cyanidin-3-o-glucoside (C3G), a type of anthocyanin, and DDP, when used alone or in combination; a BALB/c nude mouse xenograft tumour model was used. The tumour was inhibited in the three treatment groups when compared with untreated controls. The inhibition of tumour was 40.49%, 50.15%, and 58.49% when treated with C3G alone [40 mg/kg body weight (bw)], DDP alone (3 mg/kg bw), or a combination of C3G and DDP, respectively. Immunohistochemistry analysis indicated that treatment with C3G, DDP, or the combination induced apoptosis in xenograft tumours. Furthermore, after treatment, Bcl-2 level was decreased, Bax and cleaved caspase-3 expression was activated, and the PI3K/AKT/mTOR signalling pathway was modulated. These results suggest that the combination of C3G and DDP may have significant synergistic anti-tumour efficacy in patients; therefore, this combination therapy has great potential for the treatment of cervical cancer.

Introduction

Cervical cancer is a malignant tumour in the female genital organ with high incidence worldwide, seriously affecting the quality of life and health of the patient[1]. It ranks high up on the lethality list among gynaecological malignant diseases[2]. Currently, surgery, radiotherapy, and chemotherapy are the main treatment methods for early-stage cervical cancer, which can significantly prolong and improve the survival time and quality of life of most patients[3]. Chemotherapy is an important clinical treatment option for cancers, and cisplatin (DDP) is one of the most effective anti-cancer drugs to treat metastatic cervical cancer[4,5]. However, intolerance to it and adverse effects has limited its use in the clinic[6,7]. Therefore, there is still an urgent need for the development of novel effective therapeutic agents for the treatment of this aggressive malignant cancer.

In recent years, the close relationship between human health and diet has prompted us to explore bioactive chemicals in fruits and vegetables. Polyphenols, including anthocyanins, phenolic acids, and flavonoids, are the most important compounds with health benefits in fruits and vegetables[8]. Previous studies have shown that anthocyanins have a wide range of biological activities, including anti-oxidant, anti-cancer and anti-ageing[9-11]. Polyphenols interfere with carcinogenesis by targeting multiple signalling pathways and inducing apoptosis, while sparing the survival pathways in normal cells. Polyphenols are also be used as adjuvants to enhance the overall efficacy of conventional chemotherapy drugs[13]. Cyanidin-3-O-glucoside (C3G), an anthocyanin, is abundant and widely available in natural berries[12]. There is *in vivo* evidence that non-nutritional C3G exhibits numerous health-promoting effects including

anti-oxidant, cardioprotective, and anti-cancer properties[14-16]. However, the potential of C3G as a possible co-adjuvant of DDP in cervical cancer therapy is yet to be evaluated.

The phosphatidyl inositol 3-kinase (PI3K/AKT/mTOR) signalling pathway is involved in the regulation of cell proliferation, migration, and apoptosis[17]. Numerous studies have shown that the inhibition of expression of phosphatidylinositol 3-kinase (PI3K) protein may play an important role in impeding tumour growth[18,19], whereas the activation of the PI3K/AKT/mTOR pathway may be related to the serious side effects of chemotherapeutic drugs[20]. Previous studies have shown that the DDP- induced nephrotoxicity reduced by inhibiting the PI3K/AKT/mTOR pathway[21]. Therefore, it could be hypothesised that C3G combined with DDP might play a therapeutic role by regulating PI3K/AKT/mTOR pathway.

Previous studies have shown that the C3G exerted anti-tumour effects, but to the best of our knowledge, this the first time that an *in vivo* study combing C3G and DDP has been tested in a cervical cancer mouse xenograft model.

Results

The effect of C3G, DDP, or their combination on the body weight of nude mice

The change in body weight is the fundamental method to detect acute toxicity of any test substance. Generally, the acute toxicity of a drug is evaluated by examining whether the body weight of the mice decreases sharply after 72 h of treatment[22]. We proceeded to investigate the potential therapeutic benefits of C3G with DDP in vivo using mice xenograft models.

Tumors were allowed to grow for one week, after which mice were treated with C3G, DDP, or C3G+DDP for another 2 weeks (Fig. 1A). At the end of the study, we observed that DDP caused a reduction in the animal's body weight when compared with the model group ($P < 0.05$; Fig. 1B). There was no significant change in body weight in mice when we compared the C3G or C3G+DDP group, with the model group ($P > 0.05$). In addition, the mice in the C3G and the C3G+DDP groups were more lively and active than the mice in the DDP group. These results indicate that anti-cancer drug (DDP) affected the body weight in mice with tumours and that C3G alleviated, to some extent, the weight loss in tumour bearing mice.

The effect of C3G, DDP, or their combination on tumour weight and tumour growth

At the end of the experimental period, tumour tissues were excised and their volumes and weighst were measured. As shown in Figures 2A and 2B, the tumour growth was suppressed in all the treatment groups, relative to that in the model group. The tumour volumes of the combination group were significantly smaller than in the model group ($P < 0.01$), which indicates that the inhibitory effects on tumour volumes were much stronger by the combination treatment of C3G and DDP.

Tumour weight in all treatment groups decreased significantly relative to those in the model group on day 22 ($P < 0.01$; Fig. 2C). A combination of C3G (40 mg/kg/d) and DDP 3 mg/mg once in 3 days) decreased

the tumour weights form 610 ± 56.25 mg in the model group to 253.2 ± 49.51 mg ($P < 0.01$) in the treatment group. As shown in Figure 2D, compared with the model group, the tumour growth was inhibited by 40.49%, 50.15%, and 58.49%, in the C3G, DDP and C3G+DDP groups, respectively. Thus, the combination of C3G and DDP inhibited tumour growth 8.34% more than DDP alone. In summary, the combination treatment could significantly reduce tumour weight and growth, suggesting that the anti-cancer effect of the combined treatment was significantly stronger than that of DDP alone.

The effect of C3G, DDP, or their combination on cell apoptosis of the xenograft tumour

The persistence of tumour cells in the xenograft indicates that they have evaded the host immune system; therefore, an extraneous treatment is required to control uncontrolled proliferation[23]. We analysed the induction of apoptosis using the TUNEL assay, wherein fragmented DNA, a characteristic of apoptotic cells, is used to identify apoptotic cells. Cells fluorescing red owing to fragmented DNA indicates apoptotic cells. Apoptosis was observed in all treated groups, compared with that of the model group. As shown in Figure 3A, the area of fluorescence in the xenograft tumours significantly increased in mice treated with the combination of drugs (C3G + DDP) compared with that in mice treated with either C3G or DDP alone. Figure 3B shows that the percentage of cells undergoing apoptosis increased upon treatment with either C3G or DDP alone compared with the model. The percentage of apoptotic cells in the tumours of mice treated with the combination of drugs (C3G + DDP) was 15.85% more than in the tumours of mice treated with either drug alone.

The effect of C3G, DDP, or their combination on apoptotic proteins in xenograft tumour

To demonstrate the pro-apoptotic effects of C3G alone or in combination with DDP on xenograft tumour, we analysed the expression levels of Bax, Bcl-2, and cleaved caspase-3, known as apoptosis regulators. As shown in Figure 4, the xenograft tumour cells were stained with DAB that could specifically bind to Bax, Bcl-2, and cleaved caspase-3. The related proteins were imaged as blue fluorescent areas. The results indicate that the percentage of Bcl-2 protein-positive cells significantly decreased in all treatment groups compared to that of the model ($P < 0.05$; Fig. 4C and 4D). In contrast, the percentage of Bax were significantly increased by 3.77%, 3.47%, and 7.23% and those of cleaved caspase-3 by 3.03%, 2.83%, and 4.92% in the C3G, DDP and the C3G+DDP groups, respectively, compared with model group (all $P < 0.05$; Fig. 4A, 4B, 4E, and 4F).

The effect of C3G, DDP, or their combination on the phosphatidyl inositol 3-kinase PI3K/AKT/mTOR pathway in the xenograft tumour

The PI3K/AKT/mTOR pathway is involved in the regulation of cell proliferation and apoptosis[24-26]. Activation of the PI3K/AKT/mTOR signalling pathway is critical for tumour cell growth and survival in several solid cancers[27]. Therefore, to understand the molecular processes underlying the effects of C3G and DDP on xenograft tumour, we examined the expression of the signalling proteins involved in the PI3K/AKT/mTOR pathway.

The results indicate that the percentage of p-AKT, p-PI3K, and p-mTOR protein-positive cells were less in all treatment groups compared with the C3G+DDP group; less by 4.57%, 6.04%, and 6.16%, in the C3G, DDP, and C3G+DDP groups, respectively (Fig. 5). Furthermore, the percentage of these signalling-proteins-positive cells may explain why the combinational treatment exhibited stronger apoptotic effects in tumour tissues than those exhibited by the treatment with C3G or DDP alone. This study suggests that the observed apoptosis in the xenograft tumour apoptosis caused by DDP or C3G is regulated by t p-AKT, p-PI3K, and p-mTOR.

Discussion

Cervical cancer is among the most common malignant disease for women, ranking the second in the incidence list of gynaecological cancer. DDP, a classical first-generation platinum drug, is widely used as a therapy for cervical cancer. DDP resistance is related to a variety of mechanisms, such as DNA repair, increased drug inactivation (preventing drug forms from reaching their DNA targets), growth signalling pathways, and increased expression of anti-apoptotic proteins[28,29]. The application of DDP is limited owing to its adverse effects and tumours developing drug resistance[30,31].

The therapeutic index, which takes into account the toxicity of a durg at its efficacious dose, is an important factor considered while developing chemotherapy drugs. Numerous studies have looked at combining various monotherapy drugs to combat various diseases, including cancer, to improve their therapeutic index[32]. In this study, we found that C3G could enhance the efficacy of DDP by reducing the tumour weight and volume, without any observable toxicity in mice at the tested dose. These results demonstrate that C3G can improve the therapeutic effect of DDP, thereby reducing the adverse effects of DDP.

Furthermore, we studied the underlying mechanisms by which the C3G+DDP combination inhibits xenograft tumour cell growth. Apoptosis is an active process of cell death, characterised by cell contraction, chromatin aggregation with genome fragmentation, and nuclear pyknosis[33]. Promoting cancer cell apoptosis is a key characteristic of chemotherapy drugs[34]. Mitochondria are highly dynamic organelles in eukaryotic cells and play an important role in the process of apoptosis[35]. It is widely known that the Bcl-2 family of proteins play an important role in cell apoptosis and includes the pro-apoptotic Bax protein and the anti-apoptotic Bcl-2 protein[36]. Several studies have established that the down-regulated and upregulated expression of Bcl-2 and Bax, respectively, could induce mitochondrial outer membrane permeability, resulting in the release of a variety of apoptotic proteins and cell death mediators[37,38]. Caspase-3 is the main effector of programmed cell death and is known to initiate most of the apoptotic signalling cascades[39]. We found that the C3G+DDP combination could significantly induce cell apoptosis, compared with C3G or DDP alone. These findings suggest that the C3G+DDP combination has a synergistic effect as demonstrated by the increased percentage of cells expressing Bax and cleaved caspase-3 in xenograft tumorus, thereby inducing an elevated level of xenograft tumour cell apoptosis.

Numerous studies have demonstrated that the PI3K/AKT/mTOR pathway plays a central role in the growth, survival, and motility of cancer cells, making it an important target for anti-tumour drug development[40]. Inhibition of signalling along this pathway could lead to increased cell death[41]. To examine the effect of C3G on this pathway, we analysed key proteins of the PI3K/AKT/mTOR pathway in mouse cervical tumour tissue. The imbalance in the expression of PI3K/AKT/mTOR signalling proteins affects the downstream mechanisms related to tumor development. AKT, also known as protein kinase B, is a key signalling effector of PI3K and an oncogene product that regulates cell growth[42]. Upon phosphorylation-dependent activation, AKT phosphorylates its downstream substrate mTOR, thus promoting cell growth and proliferation[43], whereas inhibition of AKT phosphorylation can induce apoptosis[44]. Here, we found that the C3G+DDP combination significantly inhibited p-P13K, p-AKT, and p-mTOR expression, compared to other treatment groups.

Conclusion

Our previous studies had revealed that the combination of C3G and DDP could inhibit the growth of cervical cancer cells in culture. This study, to the best of our knowledge, is the first to investigate the effects of the combination of C3G and DDP on cervical cancer cell-induced tumour growth *in vivo*. Application of DDP together with C3G reduced tumour weight, induced apoptosis in the xenograft tumours, and down-regulated PI3K/AKT/mTOR signalling. Our data provide evidence that DDP in combination with C3G may have therapeutic potential for the treatment of cervical cancer.

Materials And Methods

Chemicals and antibodies

C3G (purity > 98%) was provided by Nanjing Plant Origin Biological Technology Co., Ltd. (Nanjing, China). DDP was purchased from Dalian Meilun Biotechnology Co., Ltd. (Dalian, China). Antibodies were purchased from Wanlei Biological Technology Co., Ltd. (Shenyang, China); detailed information on each antibody is listed in Supplementary Table S1.

Cell culture

The human cervical cancer cell line (HeLa) was obtained from Wanlei Biological Technology and cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% foetal bovine serum (FBS). Cells were maintained at 37°C in an incubator with a humidified atmosphere and 5% CO_2.

Animal origin

Twenty-four BALB/c nude mice, 5–6 weeks-old, and weighing 18 ± 2 g were provided by Beijing HFK Bioscience Co. Ltd. The mice were raised at 25 ± 2°C, with free access to feed and water. All procedures and protocols of experiments were endorsed by the Ethical Committee for the Experimental Use of Animals at Shenyang Agricultural University (approval number 202011014) under the regulations of the

“Regulations for the Administration of Affairs concerning Experimental Animals” guidelines and with the ARRIVE guidelines.

Animal model and treatments

The mice were fed for 5 days and fasted for 12 h before starting the xenograft study. HeLa cells(5×10^6) were subcutaneously injected into the right axilla to establish the mice cervical cancer model. After 24 h of tumour cell injection, mice were randomly divided into 4 groups with 6 mice per group: model group, C3G group, DDP group, and C3G+DDP group. One week after cell injection: model group, was administered gavage with 0.2 mL normal saline every day for 2 consecutive weeks; the C3G group was administered gavage with C3G [40 mg/kg body weight (bw), dissolved in distilled water] every day for 2 weeks; the DDP group, received intraperitoneal injections of DDP (3 mg/kg bw) once in three days for 2 weeks; and the C3G+DDP group was administered gavage with C3G (40 mg/kg bw) every day combined with intraperitoneal injection of DDP (3 mg/kg bw) once in three days for 2 weeks.

Measurement of tumour diameter, volume and tumour weight

After tumor formation, the diameter of the tumour growth was measured every 3 days and the volume of the tumour was calculated. Formula for tumour volume was as follows: tumor volume (mm^3) = [length diameter (mm) × short diameter (mm) × short diameter (mm)] / 2. At the end of 2 weeks of treatment, intraperitoneal infusion was performed using 10% chloral hydrate (3.5 mL/kg bw) to anaesthetise the mice, and tumours were removed for measurement of volume (mm^3) and weight (mg).

Terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labelling (TUNEL) test

TUNEL staining was performed to detect cell apoptosis in tumour tissues. The analysis was conducted as previously described[45]. Tissues were fixed in 10% formaldehyde and then embedded in paraffin blocks for sequential sectioning into 4-μm-thick sections. The tissues were washed under tap water for 4 h, subjected to a gradient ethanol series (70%, 80%, 90%, 100%, and 100%) for 1 h each, and then washed twice in PBS. Next, the tissues were treated with Proteinase K for 20 min at 37°C and washed with PBS. After drying, a 50 μL TUNEL reaction mixture was added, a cover glass was added on the slide, and the side was kept in a dark humid box at 37°C for 1 h. For the negative control group, only 50 μL fluorescein-labelled dUTP solution was added. The slides were washed thrice in PBS. After staining with DAPI for 10 min, the slides were washed thrice in PBS. A drop of PBS was added, and images were captured under the field of vision with a fluorescence microscope (BX53, Olumpus, Japan). The rate of apoptosis was calculated by determining the ratio of apoptotic cells to total cells using the Image-pro Plus 6.0 software.

Immunohistochemisitry

Immunohistochemistry was conducted as described previously[45]. Immunostaining was performed to observe and image the expression levels of Bax, Bcl-2, cleaved caspase-3, p-AKT, p-PI3K, and p-mTOR in the tumours. Brief, the tumour samples were dewaxed and hydrated at room temperature, and the

endogenous enzyme activity was inactivated and heat-antigen-repaired. The rack was replaced with a new one, the slices were hydrated, and a circle was drawn using an immunohistochemical pen. Non-specific sites were blocked, the sections were incubated with primary antibodies and washed with PBS, and then incubated with the HRP-conjugated secondary antibody (Thermo Fisher Scientific, Waltham, MA, USA). After washing with PBS and distilled water thrice successively, the sections were stained with 3, 3-diaminobenzidine (DAB) and counterstained for 3 min with haematoxylin solution. The samples were differentiated with 1% hydrochloric acid alcohol and washed with water for 20 min, dehydrated, and mounted with neutral resin. Finally, the images were analysed using Image-pro Plus 6.0 software.

Statistical analysis

The data are presented as the mean ± standard deviation (SD). The differences between samples were analysed by using one-way analysis of variance (ANOVA) using Turkey's-b comparison and considered significant at $P < 0.05$. All calculations were performed using SPSS v. 26.0 (SPSS Inc., Chicago, IL).

Declarations

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Conflict of Interest

The authors declare that they have no conflict of interest.

Authors' contributions

Xu Li: Data curation, Writing-original draft. Jingjing Mu: Data curation. Xianjun Meng: Conceptualization, Writing- review & editing.

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Figures

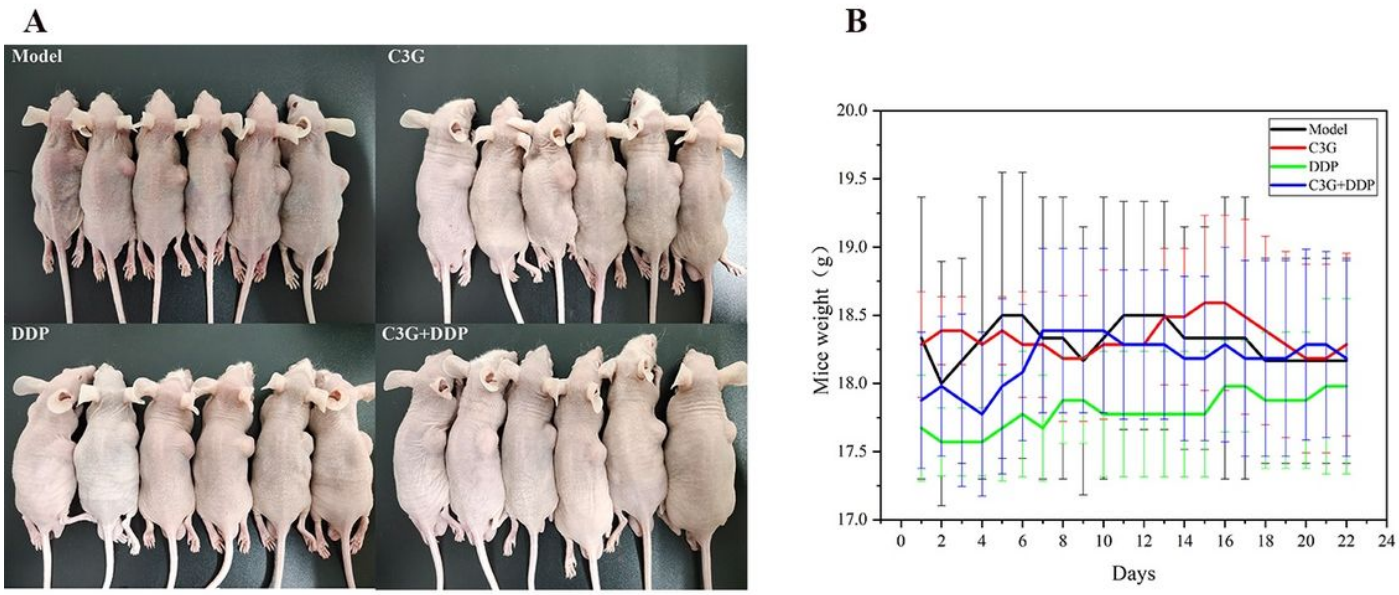

Figure 1

The effect of C3G, DDP, and their combination on body weight of nude mice. HeLa cells were injected into the right axilla of nude mice, and were treated with C3G, DDP, or combination of C3G and DDP for 2 weeks. (A) Images of in vivo tumors, n=6. (B) Body weight measured every day. Date are representative as mean ± SD.

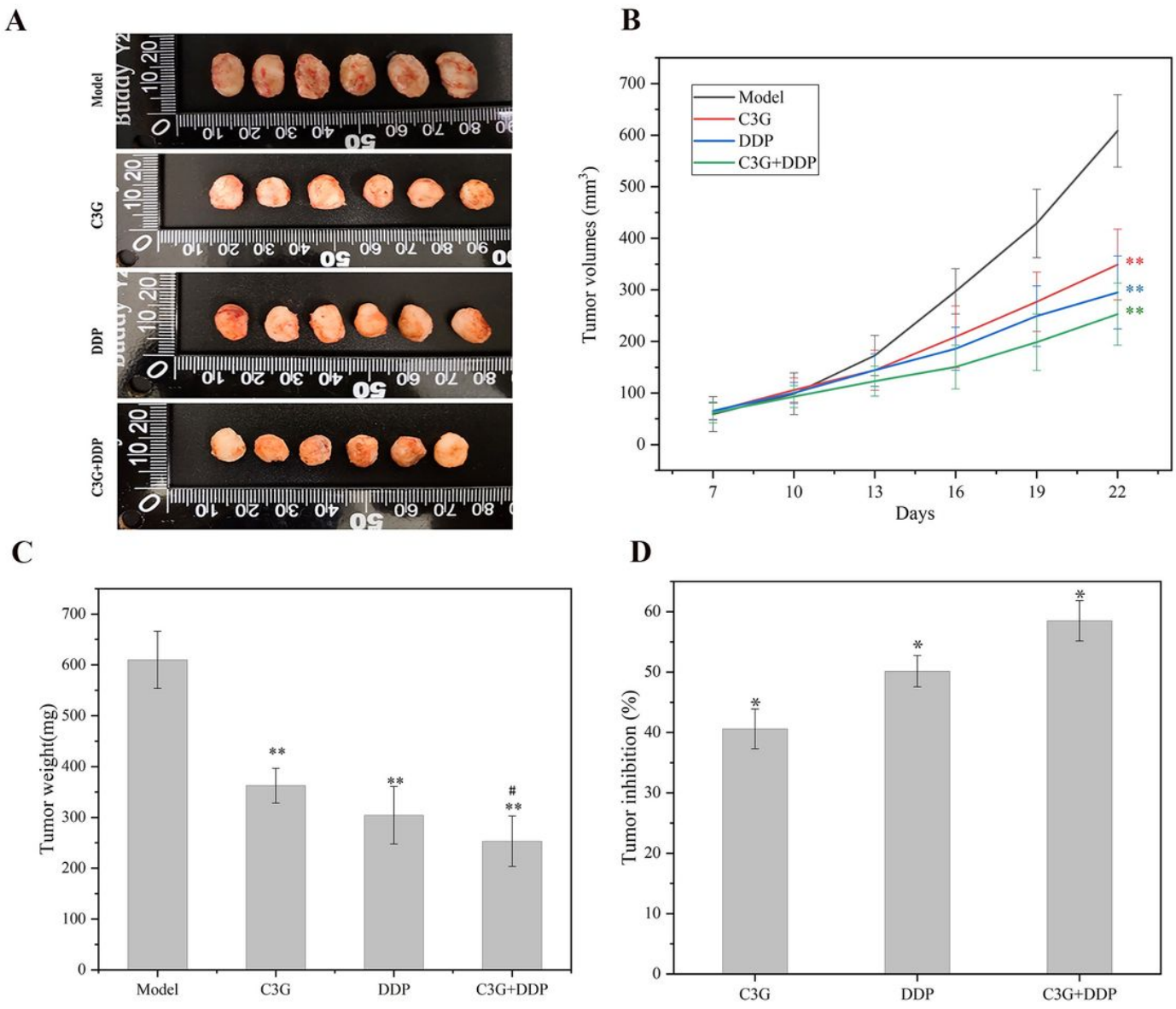

Figure 2

The effect of C3G, DDP, and their combination on tumour growth in mice. (A) Images of excised tumors, n = 6. (B) Tumour volumes were measured once every three days, n = 6. **P < 0.01 compared with the model group. (C) Tumor weight was recorded at the end of the experiment, n = 6. **P < 0.01 compared with model group.#P < 0.05 compared with C3G group. (D) Inhibition of nude mice solid tumor, n = 6. *P < 0.05 compared with model group.

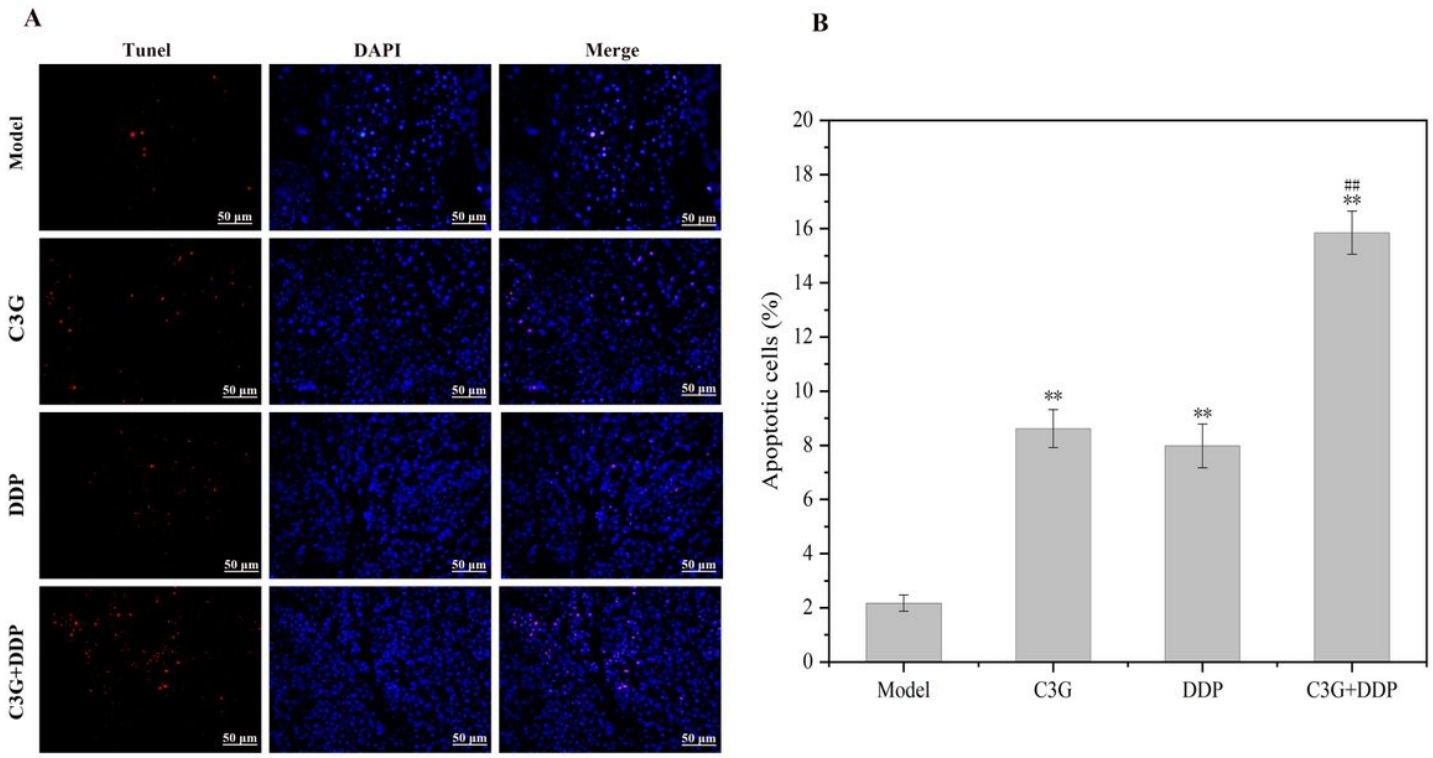

Figure 3

The effect of C3G, DDP, and their combination on apoptosis in the cervical cancer xenograft tumour. (A) The effect of treatments on tumor apoptosis (magnification × 400). (B) Quantitative analysis for apoptotic cells in tumor tissue. **P < 0.01 compared with the model group. ##P < 0.01 compared with DDP group.

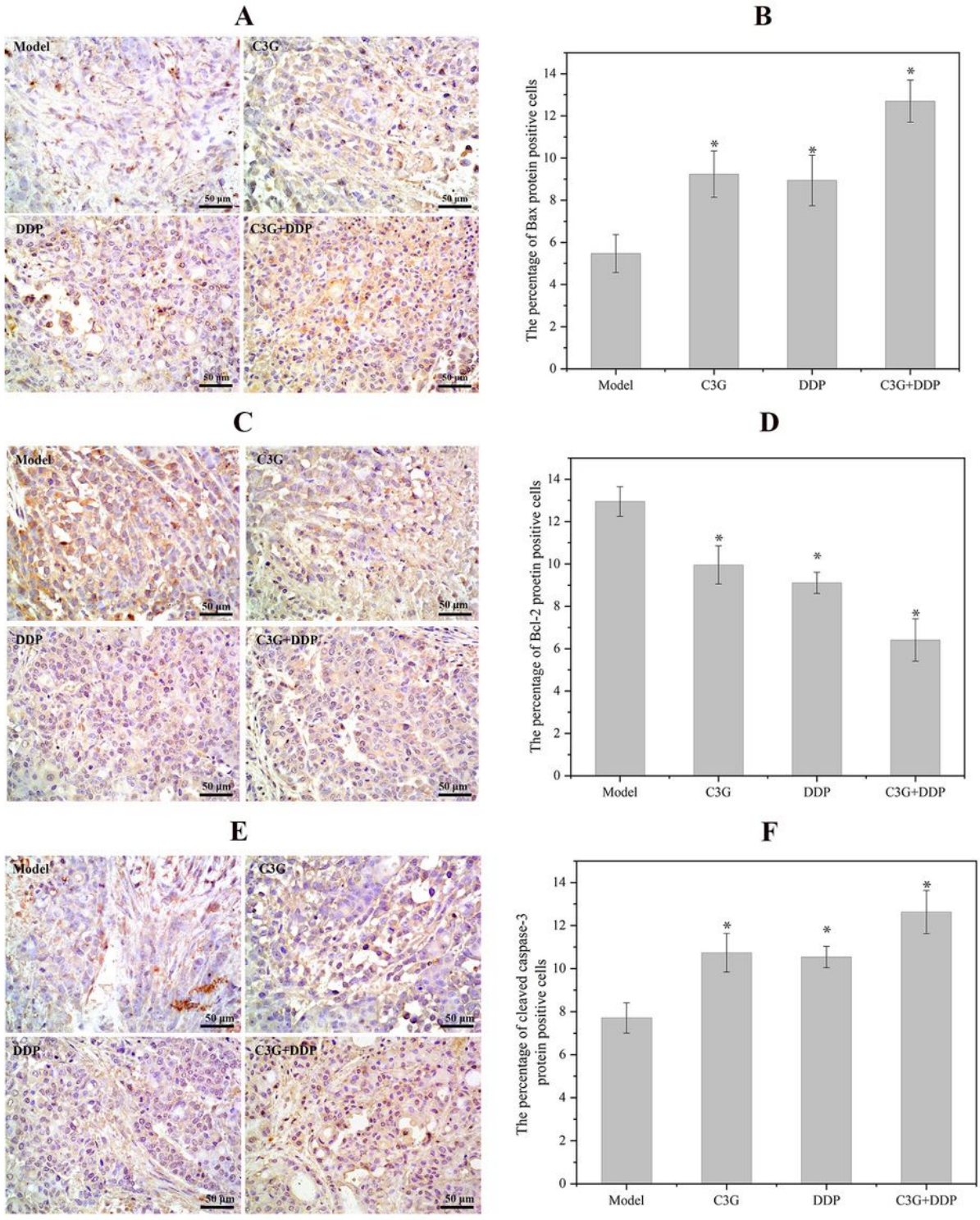

Figure 4

The effect of C3G, DDP, and their combination on apoptosis -related-protein expression in cervical cancer xenograft tumour. (A) The effect of treatments on Bax expression in xenograft tumour tissue (magnification × 400). (B) Quantitative analysis for cells positive for Bax in tumour tissue. *P < 0.05 compared with the model group. (C) The effect of treatments on Bcl-2 expression in xenograft tumour tissue (magnification × 400). (D) Quantitative analysis for cells positive for Bcl-2 in tumour tissue. *P <

0.05 compared with the model group. (E) The effect of treatments on cleaved caspase-3 expression in xenograft tumour tissue (magnification × 400). (F) Quantitative analysis for cells positive for cleaved caspase-3 in tumor tissue. *P < 0.05 compared with the model group.

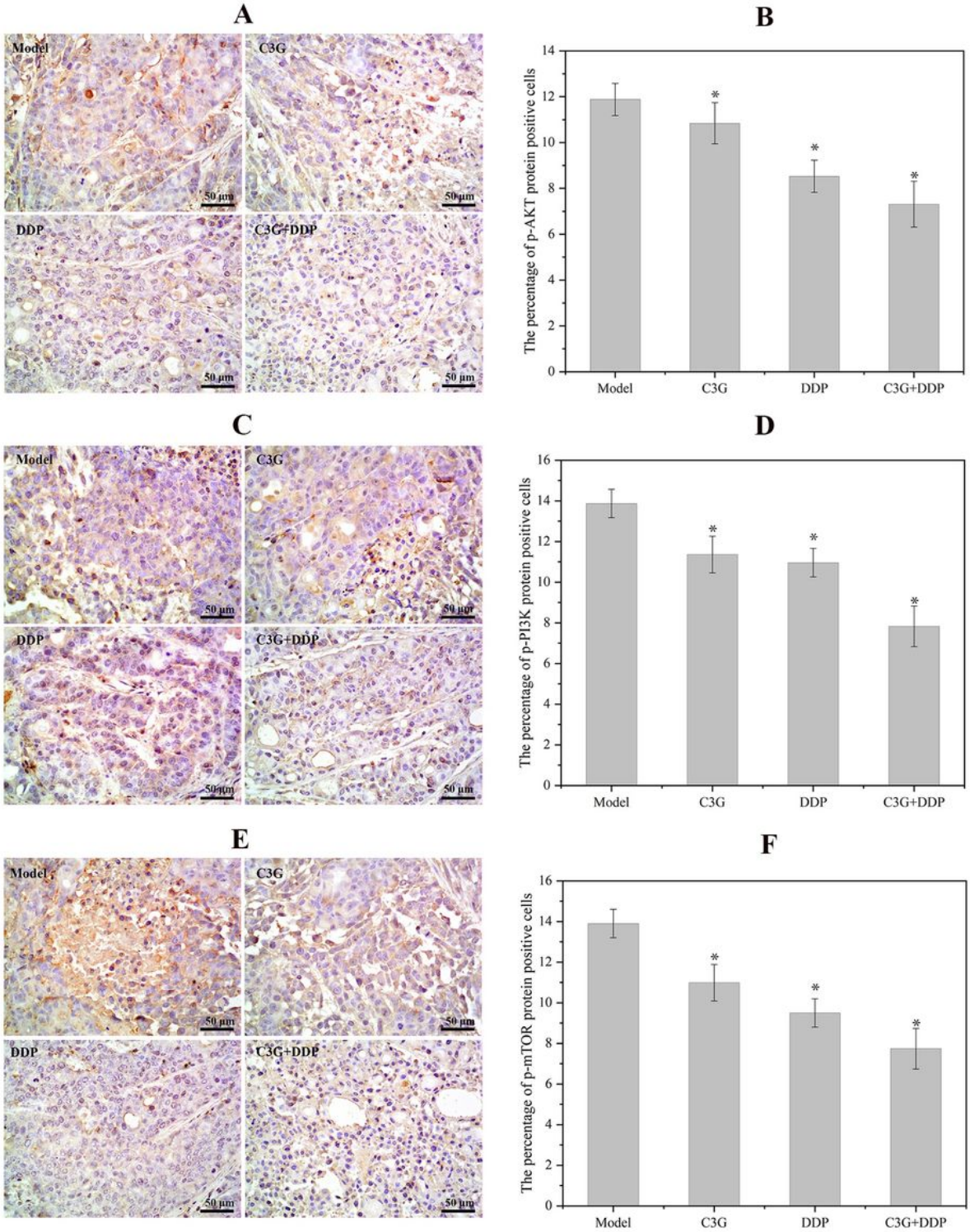

Figure 5

The effect of C3G, DDP, and their combination on the PI3K/AKT/mTOR pathway in cervical cancer xenograft tumour. (A) The effect of treatments on p-AKT expression in xenograft tumour tissue

(magnification × 400). (B) Quantitative analysis for cells positive for p-AKT in tumor tissue. *P < 0.05 compared with the model group. (C) The effect of treatments on p-PI3K expression in xenograft tumour tissue (magnification × 400). (D) Quantitative analysis for cells positive for p-PI3K in tumour tissue. *P < 0.05 compared with the model group. (E) The effect of treatments on p-mTOR expression in xenograft tumour tissue (magnification × 400). (F) Quantitative analysis for cells positive for p-mTOR in tumour tissue. *P < 0.05 compared with the model group.

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