

A novel antitumor dithiocarbamate compound inhibits the EGFR/AKT signaling pathway and induces apoptosis in esophageal cancer cells

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Abstract. Dithiocarbamate has been reported to possess a potent antitumor efficacy against several types of cancer, such as ovarian cancer, breast cancer and hepatocellular carcinoma; however, only a few studies have investigated its inhibitory effect on esophageal cancer. Dipyrldylhydrazone dithiocarbamate (DpdtC) is a novel dithiocarbamate derivative that was recently designed, synthesized and evaluated in our previous study. In the present study, the cell growth inhibition and apoptosis induced by DpdtC were measured using the CCK-8 and Annexin V-FITC/propidium iodide staining assays, respectively. Epidermal growth factor receptor (EGFR) signaling pathway and apoptosis related protein levels were examined by western blotting. *In vivo* effect of DpdtC was evaluated in nude mice bearing KYSE-450 xenograft tumors. The aims of the present study were to further evaluate the antitumor effects of DpdtC on esophageal cancer cells (KYSE-150 and KYSE-450 cells), and to investigate its potential mechanism of action *in vitro* and *in vivo*. It was found that DpdtC significantly inhibited KYSE-150 and KYSE-450 cell proliferation by regulating the EGFR/AKT signaling pathway and inducing apoptosis. In addition, this effect was further identified *in vivo*; DpdtC inhibited the growth of the KYSE-450 esophageal cancer xenografts by regulating the EGFR/AKT signaling pathway. Furthermore, DpdtC did not affect the body weight

in mice. Collectively, the present results suggested that DpdtC may be a promising antitumor drug candidate for the treatment of esophageal cancer.

Introduction

Esophageal cancer is a malignant disease associated with poor prognosis. Furthermore, esophageal squamous cell carcinoma (ESCC) is a type of esophageal carcinoma that is usually located in the upper or middle third part of the esophagus (1), and had a high-incidence rate between 90-120 per 10⁵ population in the 5-year period (1995-1999) in the Henan region in China (2).

Current therapeutic regimens for patients with esophageal cancer include surgery followed by conventional chemotherapy or radiation; however, the 5-year survival percentage remains very low at <10% (2,3). Thus, effective therapeutic agents or regimens are urgently required for improving the survival rate of patients with esophageal cancer.

Metal chelators are a class of potential therapeutics that have potent and selective anti-cancer efficacy (4,5). Most cancer cells have an increasing demand for metal ions, such as iron or copper, to maintain an appropriate proliferation rate; therefore, chelators may be a potential treatment regimen for treating cancer types (6,7). Previous studies have reported that metal chelators, such as di-2-pyridyl ketone-4,4-dimethyl-3-thiosemicarbazone (Dp44mT), possess an inhibitory effect against colon cancer and prostate cancer (8,9).

Dithiocarbamates are sulfur-containing compounds with an excellent chelating property toward metal ions that can regulate crucial molecules involved in reactive oxygen species accumulation, cell cycle arrest, apoptosis or autophagy (6,10,11). However, the molecular targets of numerous dithiocarbamates, such as di-2-pyridylhydrazone dithiocarbamate and pyrrolidine dithiocarbamate remain unknown. Dixon *et al* (8) revealed that Dp44mT may exert its anti-growth activity by inhibiting the oncogenic ERK1/2 signaling pathway. Moreover, Chen *et al* (9) reported that the iron chelator desferrioxamine (DFO) can inhibit epithelial-mesenchymal transition (EMT) induced by the transforming growth factor- β and by elevating the protein expression of N-myc downstream-regulated gene 1.

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Our previous study synthesized a class of dithiocarbamate derivative, dipyridylhydrazone dithiocarbamate (DpdtC), and assessed its anti-cancer activity on hepatocellular carcinoma cells (6). It was revealed that DpdtC downregulates erb-b2 receptor tyrosine kinase 2 (ERBB2) expression and disrupts the formation of a heterodimer between ERBB2 and epidermal growth factor receptor (EGFR), which further resulted in the inactivation of ERBB2/ERK 1/2 signaling in ERBB2-overexpressed ovarian cancer cells (12).

The EGFR/AKT signaling pathway has an important role in the growth and proliferation of esophageal cancer cells (13). In the present study, the antitumor effects of DpdtC on esophageal cancer cells were evaluated and its potential mechanism of action was investigated, which may be associated with EGFR/AKT signaling pathway inhibition. The present study aimed to identify the potential of DpdtC as a drug candidate for treatment of EGFR-positive esophageal cancer types, which will aid in the clinical development of esophageal cancer treatment.

Materials and methods

Cell lines and animals. The human esophageal cancer cell lines KYSE-150 and KYSE-450 were purchased from the American Type Culture Collection. Cells were cultured with RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% Fetal Bovine Serum (Gibco; Thermo Fisher Scientific, Inc.), 2 mmol/l glutamine, 100 IU/ml penicillin and 100 mg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) in an incubator at 37°C with 5% CO₂ for 48 h. Female BALB/c nude mice (age, 5 weeks; weight, 16–19 g; n=15) were obtained from the Beijing Vital River Laboratory Animal Technology Co., Ltd. All animals were treated in accordance with guidelines of the Committee on Animals of the Xinxiang Medical University and was approved by Biomedical Ethics Committee of Xinxiang Medical University.

In vitro cytotoxicity assays. Firstly, the effects of different treatment times (24, 48 or 72 h) on the cytotoxicity of DpdtC (Henan International Joint Lab of Recombinant Protein) in esophageal cancer cells was assessed for determining the appropriate treatment time. Esophageal cancer KYSE-150 and KYSE-450 cell lines were treated with 10 µM DpdtC for the aforementioned 3 time points (24, 48 or 72 h) at 37°C with 5% CO₂. Cell viability was then tested using the Cell Counting 8 (CCK-8) Kit (Dojindo Molecular Technologies, Inc.) according to the manufacturer's instructions. Next, cells were treated with DpdtC (Henan International Joint Lab of Recombinant Protein) at a series of concentrations, which was diluted from 50 µM in a 2X dilution manner (50, 25, 12.5, 6.25, 3.125, 1.5625 and 0.78125 µM) at 37°C with 5% CO₂ for 48 h. After 2 days, cell viability was determined using CCK-8 Kit (Dojindo Molecular Technologies, Inc.). The percentage of surviving cells was calculated using the following formula: [(A450 of experiment-A450 of background)/(A450 of untreated control-A450 of background)] x100. The well treated with only medium without DpdtC was the untreated control. IC₅₀ was calculated using non-linear regression analyses utilizing GraphPad Prism 5 software (GraphPad Software, Inc.).

In vivo therapy study. All animal experimentation followed internationally recognized Animal Research: Reporting of *in vivo* Experiments guidelines (14). Female BALB/c nude (age, 5 weeks) mice were maintained at 22±2°C and 50–60% humidity in a 12 h dark/light cycle, with continuous free access to food and water.

KYSE-450 cells (5x10⁶ per mouse) were inoculated subcutaneously into the right flank of the female BALB/c nude mice. When tumor volumes reached an average of ~150 mm³, the mice were randomly divided into 3 groups (n=5 in each group): i) A PBS-treated group as control; ii) a DpdtC-treated group at a low dose (1 mg/kg); and iii) a DpdtC-treated group at a high dose (3 mg/kg). Mice were intraperitoneally injected with 200 µl 10 mM PBS or DpdtC (1 or 3 mg/kg) four times (day 0, 2, 4 and 6) as indicated in the period of 14 days. The dosages of DpdtC were chosen according to our previous study (12). On day 13 post-first injection, the mice were euthanized using carbon dioxide gas (20%/min gradual displacement) and monitored for 5 min to confirm cardiac arrest, and the tumors were removed for subsequent western blot examination. The tumors were measured with digital calipers, and tumor volumes were calculated using the formula: Volume=Length x (Width)²/2. Unspecific toxicity evaluation was determined in tumor-bearing nude mice injected with PBS control or DpdtC by monitoring the body weight of these mice at regular intervals during the whole therapeutic period.

Immunoblotting. Western blot analysis was performed according to previously described procedures (15). The sample was the protein extracted from cell pellets or tumor tissues. Cells were treated with DpdtC (0, 10, 20 or 30 µM) for 24 h before being lysed. Tumor tissues were removed on day 13 post the first administration of DpdtC. Then, cells or tumor tissues were lysed in lysis buffer [50 mmol/l Tris-HCl pH 7.4, 150 mmol/l NaCl, 0.1% SDS, 1% Triton x-100 and 0.5% deoxycholic acid sodium salt (w/v)] supplemented with 2 µl/ml protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA) on ice for 30 min and centrifuged at 12,000 x g, 4°C for 20 min to remove cell debris. Subsequently, the protein (20 µg/well) from the cell lysates were separated using a 10% SDS-PAGE gel and transferred to PVDF membrane for blocking in 5% skimmed milk for 1 h 30 min at room temperature. Next, the membrane was immunoblotted with respective antibodies against EGFR (1:1,000; cat. no. 54359; Cell Signaling Technology, Inc.), phosphorylated (p)-EGFR (1:1,000; cat. no. 2234; Cell Signaling Technology, Inc.), AKT (1:1,000; cat. no. 10176-2-AP; ProteinTech Group, Inc.), p-AKT (1:2,000; cat. no. 4060; Cell Signaling Technology, Inc.), Poly (ADP-ribose) polymerase (PARP; 1:500; cat. no. BM5118; Boster Biological Technology), BAX (1:1,000; cat. no. 50599; ProteinTech Group, Inc.), Bcl-2 (1:1,000; cat. no. 4223; Cell Signaling Technology, Inc.), Bcl-2-binding component 3, isoformed 1/2 (BBC3; 1:1,000; cat. no. 55120; ProteinTech Group, Inc.) or β-actin (1:5,000; cat. no. ab179467; Abcam), and subsequently with horseradish peroxidase-conjugated goat anti-mouse/rabbit secondary antibodies (1:5,000; cat. no. SA00001-1 or SA00001-2; ProteinTech Group, Inc.) at room temperature for 45 min. After washing the membrane with wash buffer (Tris buffered saline with Tween-20, pH 8.0),

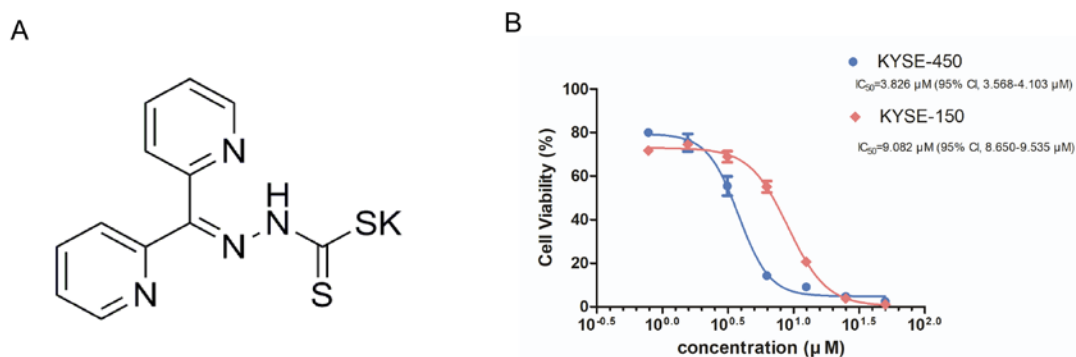


Figure 1. DpdtC markedly reduces the proliferation of esophageal cancer cells *in vitro*. (A) Chemical structure of DpdtC. (B) Effects of DpdtC on the cytotoxicity of KYSE-450 and KYSE-150 cells. Cells were treated with increasing concentrations of DpdtC (0.78125, 1.5625, 3.125, 6.25, 12.5, 25 and 50 μ M) for 48 h and then viabilities were determined using Cell Counting Kit-8 assay. All samples were tested in triplicate. Data are presented as the mean \pm SD. DpdtC, Dipyrldylhydrazone dithiocarbamate.

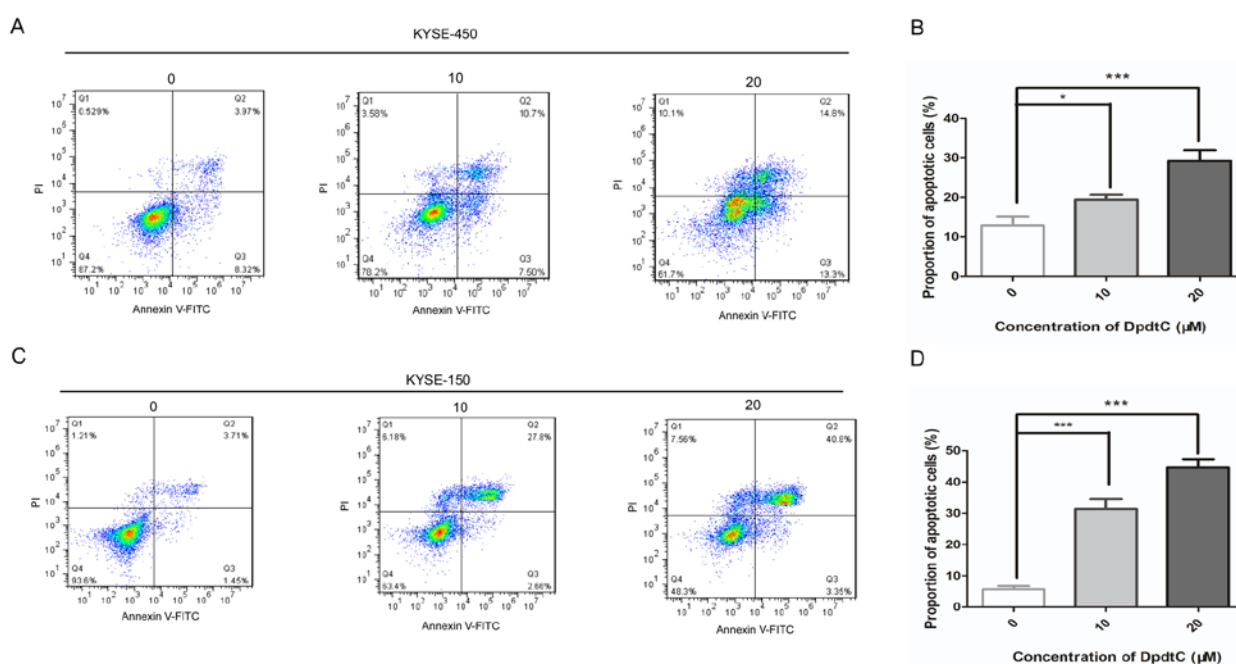


Figure 2. DpdtC induces apoptosis in both KYSE-450 and KYSE-150 cells. The number of apoptotic KYSE-450 cells following treatment with DpdtC (0, 10 or 20 μ M) for 20 h was determined using (A) flow cytometry and the results were analyzed (B) statistically. (C) The number of apoptotic KYSE-150 cells following treatment with DpdtC (0, 10 or 20 μ M) for 20 h was determined using flow cytometry and the results were analyzed (D) statistically. Data are presented as the mean \pm SD. * P <0.05, *** P <0.001. DpdtC, Dipyrldylhydrazone dithiocarbamate; PI, propidium iodide.

the bands were detected using the sensitive ECL reagent (GE Healthcare Life Sciences), and visualized using a ChemiDoc imaging system (Bio-Rad Laboratories, Inc.). Relative densitometry analysis of protein expression level was normalized to control β -actin antibody using Image J software v.1.46 (National Institute of Health).

Apoptosis analysis. Apoptosis analysis was performed as previously described (16). For flow cytometry analysis, KYSE-450 or KYSE-150 cells (1×10^6 /well) were plated in 6-well plate and treated with DpdtC (0, 10 or 20 μ M) for 20 h at 37°C. The cells were then labeled with Annexin V (20 μ g/ml) and propidium iodide (PI) (50 μ g/ml) (Dojindo Molecular Technologies, Inc.) at room temperature for 15 min. Apoptotic rates were analyzed using a FACSCalibur flow cytometer (BD Biosciences) and

calculated using FlowJo software v.7.6.1 (Treestar, Inc.). The rate of early apoptosis was calculated by Annexin V (+) and PI (-), while the rate of late apoptosis was calculated by Annexin V (+) and PI (+).

Statistical analysis. Statistical analysis was performed with GraphPad Prism software v.5.0.1 (GraphPad Software, Inc.). All experiments were repeated 3 times. Numerical values were expressed as the mean \pm standard deviation. For the *in vitro* and *in vivo* studies, the differences between the groups were analyzed using one-way ANOVA analysis with a Dunnett's Multiple Comparison post-hoc Test. One-way ANOVA analysis with a Tukey's Multiple Comparison Test was used for analysis of data in Fig. S1. P <0.05 was considered to indicate a statistically significant difference.

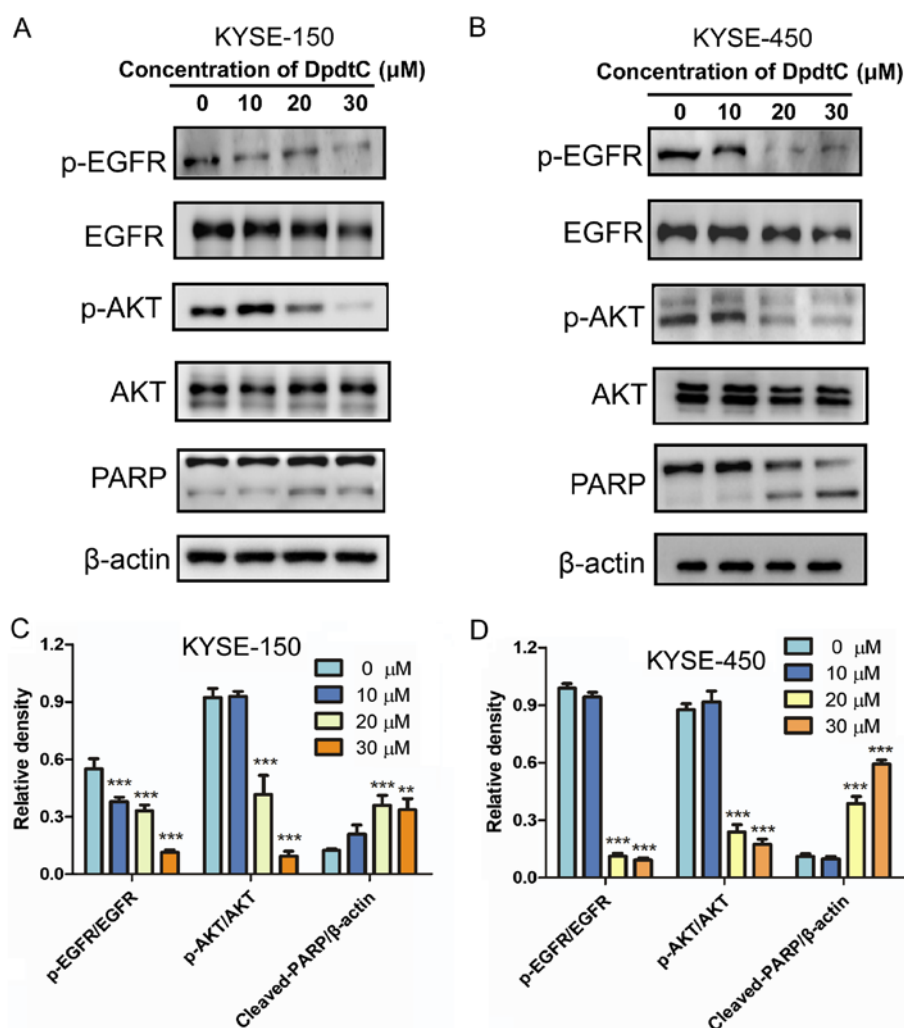


Figure 3. DpdtC inhibits the EGFR/AKT signaling pathway in KYSE-150 and KYSE-450 cells. (A) KYSE-150 and (B) KYSE-450 cells were treated with DpdtC (0, 10, 20 or 30 μ M) for 24 h before being evaluated. EGFR, p-EGFR, AKT, p-AKT and PARP were analyzed by immunoblotting assay. Quantification of protein signal intensity in (C) KYSE-150 and (D) KYSE-450 cells and expressed relative to the β -actin or total EGFR/AKT protein using ImageJ software. Data are presented as the mean \pm SD of three independent experiments. ** $P < 0.01$, *** $P < 0.001$ vs. untreated control cells. DpdtC, Dipyrldylhydrazone dithiocarbamate; p-, phosphorylated; PARP, Poly (ADP-ribose) polymerase; EGFR, epidermal growth factor receptor.

Results

DpdtC exhibits growth inhibitory effects against esophageal cancer cells in vitro. The cytotoxicity of DpdtC against EGFR-overexpressed KYSE-150 and KYSE-450 cells was examined (2,13). The chemical structure of DpdtC is presented in Fig. 1A. Based on the preliminary experiments, 48 h treatment time with DpdtC was selected for the following CCK-8 assay (Fig. S1). It was demonstrated that DpdtC had a concentration-dependent inhibitory effect in both KYSE-150 and KYSE-450 cells treatment for 48 h. IC_{50} for KYSE-450 and KYSE-150 cells was 3.826 μ M (95% CI, 3.568-4.103 μ M) and 9.082 μ M (95% CI, 8.650-9.535 μ M), respectively (Fig. 1B).

DpdtC induces apoptosis in both KYSE-150 and KYSE-450 cells. Subsequently, whether DpdtC induces apoptosis was investigated in KYSE-150 and KYSE-450 cells. The percentage of apoptotic cells was determined using flow cytometry following Annexin V and PI staining. The results suggested that the apoptotic percentage was significantly increased in both KYSE-150 and KYSE-450 cells treated

with an increasing concentration of DpdtC (Fig. 2). Moreover, a significant cleavage of PARP, a classical apoptotic initiator, was observed in both cancer cells treated with DpdtC (Fig. 3). Interestingly, KYSE-450 cells appeared to be more sensitive to DpdtC treatment in the cytotoxicity assay, while the apoptosis percentage of KYSE-450 cells was lower compared with that in the KYSE-150 cells at the same concentration of DpdtC (Figs. 1 and 2).

DpdtC inhibits the EGFR/AKT signaling pathway in KYSE-150 and KYSE-450 cells. The expression of EGFR is increased in esophageal cancer cells KYSE-150 and KYSE-450 (2,13). In the present study, a significant decrease in the phosphorylation levels of EGFR and AKT was identified in both KYSE-150 and KYSE-450 cells treated with DpdtC. Moreover, this decrease was dose-dependent (Fig. 3).

DpdtC inhibits the growth of KYSE-450 tumor xenografts. To evaluate the inhibitory activity of DpdtC *in vivo*, the therapeutic effect of DpdtC was examined in nude mice with KYSE-450 xenograft tumors. It was found that DpdtC significantly

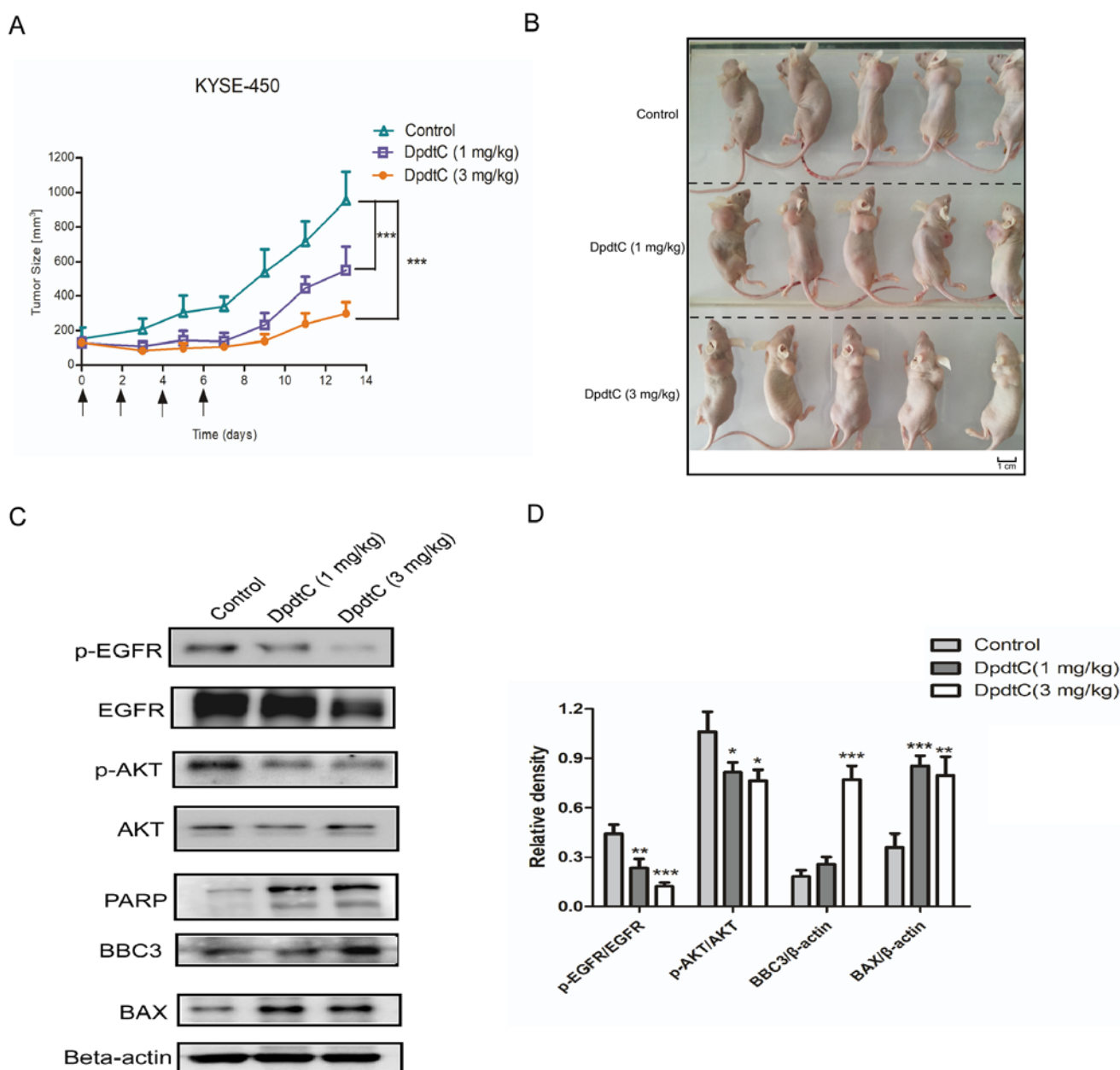


Figure 4. *In vivo* effects of DpdtC in the KYSE-450 tumor-bearing nude mice. (A) Mean tumor volumes of mice xenografted with KYSE-450 cells and treated with different doses of DpdtC (1 or 3 mg/kg). $n=5$. The black arrows indicate the DpdtC treatment times. (B) KYSE-450 tumor-bearing mice were imaged and tumors were removed on day 13 post-administration. Tumor tissues were isolated from KYSE-450 xenografts following treatment with control (PBS) or DpdtC (1 or 3 mg/kg), then the expression levels of EGFR, p-EGFR, AKT, p-AKT, PARP, BBC3, BAX and β -actin were determined using (C) western blot analysis and the results were (D) quantified and the protein levels expressed relative to the β -actin or total EGFR/AKT protein using ImageJ software. Data are presented as the mean \pm SD of three independent experiments. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. control group. DpdtC, Dipyrityldihydrazone dithiocarbamate; p-, phosphorylated; PARP, Poly (ADP-ribose) polymerase; EGFR, epidermal growth factor receptor; BBC3, Bcl-2-binding component 3.

decreased tumor growth compared with that in the control-PBS treatment group (Fig. 4A and B). Moreover, while both concentrations of DpdtC reduced the growth of tumors, the reduction was higher with 3 mg/kg DpdtC. In addition, DpdtC treatment did not result in loss in body weight (Fig. S2). Collectively, the results suggested that DpdtC had a dose-dependent inhibitory effect against KYSE-450 esophageal cancer *in vivo*.

DpdtC induces apoptosis and inhibits the EGFR/AKT signaling pathway *in vivo*. To further assess the mechanism of action of DpdtC *in vivo*, tumor samples from treated mice were collected and analyzed using an immunoblotting assay. Related apoptotic

markers, including PARP, BBC3 and BAX were also examined to evaluate the apoptosis level *in vivo*. It was found that the expression levels of p-EGFR and p-AKT in tumors were significantly decreased compared with that in the control-PBS treatment group. Furthermore, PARP was significantly cleaved, while BBC3 and BAX expression levels were significantly elevated in the DpdtC-treated groups (Fig. 4C and D).

Discussion

Metal chelators possess a potent and targeted antitumor activity against a variety of types of cancer including myeloid

leukemia, hepatocellular carcinoma and breast cancers etc. (6,17-19). Previous studies have reported that Dp44mT or DFO may inhibit cancer cell proliferation by regressing multiple signaling pathways including transforming growth factor- β , AKT, ERK and c-Abelson murine leukemia viral oncogene homolog 1-adaptor molecule CrkII pathways related to tumor progression and metastasis (4,20,21). Dithiocarbamates are sulfur-containing compounds with a strong chelating ability toward metal ions (11,22). Moreover, their derivatives, such as the synthetic gold(III) dithiocarbamate and pyrrolidine derivative of dithiocarbamate may be inhibitors targeting NF- κ B (23), inhibitors against proteasome (24), DNA intercalators (25) and inactivators of metal-containing enzymes (26); however, the underlying mechanism remains to be elucidated.

Our previous study characterized and assessed the chemical properties of DpdtC (6). To the best of our knowledge, the present study was the first to examine the antitumor efficacy of DpdtC against esophageal cancer cells and to investigate its mechanism of action. The present results suggested that DpdtC exhibited effective antitumor effects by inhibiting the EGFR/AKT signaling pathway and inducing apoptosis of esophageal cancer cells *in vitro* and *in vivo*. Furthermore, it was found that DpdtC induced the downregulation of EGFR at a relatively high concentration (30 μ M) *in vitro* or at a high dose (3 mg/kg) *in vivo*, which suggested that the growth inhibition caused by DpdtC may be associated with EGFR downregulation. In addition, the results suggested that treatment with DpdtC was well tolerated by the mice, without affecting their body weights. Therefore, it was hypothesized that DpdtC exerted its antitumor effects primarily by suppressing the EGFR/AKT signaling pathway. DpdtC has a relative weaker potency on inducing apoptosis in KYSE-450 cells compared with that in the KYSE-150 cells; however, it may more effectively inhibit the phosphorylation of EGFR and AKT in KYSE-450 cells compared with that in KYSE-150 cells. Collectively, the results suggested that DpdtC may exert its effects by inhibiting the EGFR/AKT signaling pathway and inducing apoptosis, thus suggesting the potential of DpdtC as a drug candidate for the treatment of EGFR-positive esophageal cancer types.

Currently, esophageal cancer lacks potent treatment regimens, and the 5-year survival rate is <10%, thus the development of more effective therapeutic agents is required (3). Our previous study developed a novel EGFR-targeted antibody-drug, denoted as PT that exerts antitumor effects on esophageal cancer types by inhibiting the EGFR/ERK1/2 pathway and inducing apoptosis via blockade of the nuclear factor erythroid 2-related factor 2/Kelch-like ECH-associated protein 1 pathway (13). The present study evaluated *in vivo* the novel drug, DpdtC which has the potential to be used in clinical treatment. Moreover, we hypothesized that the combination of EGFR-targeted antibody drugs with DpdtC could achieve greater antitumor effects by synergistically inhibiting the EGFR downstream signaling pathway and inducing apoptosis in esophageal cancer types, which will be further investigated in future studies.

In conclusion, the present study identified a promising anti-cancer agent, DpdtC, which targets the EGFR/AKT

pathway and induces apoptosis, and thus has potential to be a novel drug candidate in treating esophageal cancer types.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YY and SD designed the study. YY, ZT, XZ and YL collected the data and performed the experiments. YY and SD performed the statistical analyses. YY and SD wrote and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were approved by the Biomedical Ethics Committee of Xinxiang Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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