

# Niclosamide inhibits the cell proliferation and enhances the responsiveness of esophageal cancer cells to chemotherapeutic agents

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**Abstract.** Niclosamide is an FDA-approved anthelmintic drug, and may elicit antineoplastic effects through direct STAT3 inhibition, which has been revealed in numerous human cancer cells. Chemotherapy is the standard treatment for advanced esophageal cancers, but also causes severe systemic side effects. The present study represents the first study evaluating the anticancer efficacy of niclosamide in esophageal cancers. Through western blot assay, it was demonstrated that niclosamide suppressed the STAT3 signaling pathway in esophageal adenocarcinoma cells (BE3) and esophageal squamous cell carcinoma cells (CE48T and CE81T). In addition, niclosamide inhibited cell proliferation as determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay and soft agar

colony forming assay, and induced cell apoptosis as determined by Annexin V and PI staining. The induction of p21 and G1 arrest of the cell cycle also was revealed in niclosamide-treated CE81T cells by qPCR and flow cytometric assays, respectively. Furthermore, in the combination analysis of niclosamide and chemotherapeutic agents by MTS assay, low IC<sub>50</sub> values were detected in cells co-treated with niclosamide, with the exception of cisplatin-treated CE81T cells. To confirm the results using an apoptosis assay, the apoptotic enhancement of niclosamide was only demonstrated in CE48T cells co-treated with 5-FU, cisplatin, or paclitaxel, and in BE3 cells co-treated with paclitaxel, but not in CE81T cells. These findings indicate a future clinical application of niclosamide in esophageal cancers.

## Introduction

In 2018, esophageal cancer ranked seventh for cancer incidence and sixth as the leading cause of cancer-related mortality in the world, and ~70% of cases occurred in men. In men, the rates were also 2-fold higher in Human Development Index (HDI) countries, with mortality rates ranking fifth in these countries (1). Esophageal squamous cell carcinomas (ESCC) and esophageal adenocarcinomas (EAC) account for >95 % of the various esophageal malignancies. Moreover, the incidence of EAC is rapidly increasing in the United States and Western Europe (2). Esophagectomy alone is associated with a high rate of recurrence and low 5-year survival rates. A recent clinical trial revealed that neoadjuvant chemotherapy significantly improved survival in patients with resectable tumors (3). Conventional chemotherapeutic agents can effectively kill actively proliferating cells, however, the non-selective targeting can cause the destruction of both healthy and cancerous cells. Ultimately, chemotherapy leads to multiple side effects and the occurrence of drug resistance in patients. Therefore, an important approach is to identify low-toxicity and non-cancer agents for combination therapy in order to reduce the dose requirement of chemotherapeutic agents (4). Drug repurposing is an efficient approach, using clinically-approved drugs for

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*Abbreviations:* AML, acute myelogenous leukemia; AR, androgen receptor; EAC, esophageal adenocarcinoma; EGFR, epidermal growth factor receptor; ER, estrogen receptor; ESCC, esophageal squamous cell carcinoma; 5-FU, 5-fluorouracil; FDA, Food and Drug Administration; IC<sub>50</sub>, half maximal inhibitory concentration; IL6, interleukin 6; LRP6, lipoprotein receptor-related protein 6; mTOR, mammalian target of rapamycin; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; NF-κB, nuclear factor-κB; PI, propidium iodide; PI3K, phosphoinositide 3 kinase; ROS, reactive oxygen species; STAT3, signal transducer and activator of transcription 3; WNT, wingless and int-1

*Key words:* esophageal cancer, niclosamide, chemotherapy, apoptosis, cell cycle arrest

different disease treatments bypassing the long streamline of the drug discovery process (5). In order to enhance the killing efficacy of cancer cells and ameliorate the side effects caused by cancer treatments, it is significant to study and evaluate both the feasibility and efficacy of combination therapy with chemotherapeutic agents plus repurposed non-cancer drugs targeting similar pathways found in cancer (6). Based on this concept, searching for non-cancer clinical medications and evaluating the feasibility of combination therapy of these non-cancer clinical medications and chemotherapeutic agents for esophageal cancers is the focus of this research.

Niclosamide is an FDA-approved chewable tablet consumed orally against tapeworms and schistosomiasis. The mechanism of niclosamide action is through the inhibition of glucose uptake, oxidative phosphorylation, and anaerobic metabolism in the target worms (7). Except for antiprotozoal function, the antineoplastic effects of niclosamide has been revealed in many human cancer cells, including adrenocortical carcinoma (8), breast (9-11), colorectal (12,13) and cervical cancer (14), glioblastoma (15), hepatocellular carcinoma (16,17), head and neck (18,19) and lung cancer (20,21), leukemia (22,23), nasopharyngeal cancer (24), osteosarcoma (25), oral squamous cell carcinoma (26,27), ovarian (28-30), prostate (31-33), renal (34) and thyroid cancer (35). Niclosamide treatment was reported to induce apoptosis of cancer cells *in vitro* (7,10,23) and suppress tumor size in animal studies (11,29). Moreover, the combination of anticancer agents with niclosamide synergistically suppressed cell proliferation of acute myelogenous leukemia, head and neck, ovarian, prostate and non-small lung cancer (19,21,23,30,31). However, whether niclosamide is effective against esophageal cancer has not been investigated yet.

The molecular mechanisms underlying the antineoplastic effect of niclosamide have been explored in many human malignant cancers, indicating that niclosamide exhibits anticancer activity by suppressing many oncogenic signaling pathways concurrently (7,13,17,23,27,28,30,36). For instance, niclosamide has been identified as a direct inhibitor of signal transducer and activator of transcription 3 (STAT3) through interaction with the DNA-binding domain (37). In ovarian cancer, niclosamide significantly decreased the expression of proteins in the wingless/integrated (Wnt), mammalian target of rapamycin (mTOR) and STAT3 pathways and caused significant inhibition of proliferation of cells (28). In acute myeloid leukemia, niclosamide could induce apoptosis of AML blast cells through inhibition of the nuclear factor- $\kappa$ B (NF- $\kappa$ B) pathway and increasing the production of reactive oxygen species (23). In lung and head and neck cancers, niclosamide suppressed erlotinib-induced STAT3 phosphorylation, and a combination of erlotinib and niclosamide decreased tumor size in animal model experiments (19,21). In advanced prostate cancer, niclosamide blocked the interleukin 6 (IL6)/STAT3/androgen receptor (AR) pathway to overcome enzalutamide resistance and inhibit migration and invasion (31).

In the present study, the antineoplastic effects of niclosamide on esophageal cancer cells were investigated and it was revealed that niclosamide suppressed the STAT3 signaling pathway and inhibited cell proliferation in esophageal cancer cells. Niclosamide also induced cell apoptosis and G1-phase

arrest of the cell cycle. Furthermore, the combination treatment of niclosamide and chemotherapeutic drugs selectively reduced the dose requirement of the chemotherapeutic drugs in order to obtain the IC<sub>50</sub> efficacy. These findings indicated that niclosamide may be used as a single or combined drug treatment for esophageal cancer.

## Materials and methods

**Reagents.** Niclosamide (product no. N3510), 5-fluorouracil (5-FU) (product no. F6627), cisplatin (P4394), and paclitaxel (T7402) were purchased from Sigma-Aldrich (Merk KGaA). Cisplatin was dissolved in ddH<sub>2</sub>O, whereas, niclosamide, 5-FU and paclitaxel were dissolved in dimethyl sulfoxide (DMSO). The solvent was routinely used in the control group of the experiment.

**Cell culture.** Esophageal cancer cell lines, BE3 (adenocarcinoma), CE48T/VGH and CE81T/VGH (squamous cell carcinoma) were courtesy of Dr Yen (38) and Dr Lee (39), respectively. BE3 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) and CE48T and CE81T were cultured in Roswell Park Memorial Institute (RPMI) 1640 (Gibco BRL; Thermo Fisher Scientific, Inc.), supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin solution (Gibco-BRL; Thermo Fisher Scientific, Inc.). The cells were grown in a humidified incubator containing 5% CO<sub>2</sub> at 37°C.

**MTS assay.** To determine the cytotoxicity of niclosamide and the combined effect of niclosamide and chemotherapeutic agents, cells were seeded in 96-well plates overnight and treated for 72 h with different concentrations of niclosamide (1.25-20  $\mu$ M) or co-treated with 2.5  $\mu$ M niclosamide and serial doses of chemotherapeutic drugs, or DMSO as a vehicle control. The cell viability was then evaluated by MTS assay. Twenty microliters of the MTS CellTiter 96 Cell Proliferation Assay reagent (Promega Corporation) were added to each well. After 2 h of incubation at 37°C, the absorbance of the colored product was measured on an EPOCH2 microplate reader (BioTek Instruments, Inc.).

**Colony-forming assay.** Esophageal cancer cell lines to be tested were trypsinized and dissociated into single-cell suspensions for plating in 6-well plates. An agar suspension (0.3% agar) containing colony-forming cells ( $2 \times 10^4$  cells/well) is plated over an agar underlay (0.6% agar). Niclosamide (2.5  $\mu$ M) was delivered to the cells. Tissue culture medium was replenished every 3 days. After 21 days of culture, the cells were washed twice with phosphate-buffered saline (PBS) and incubated in fresh medium with MTS reagent for 2 h at 37°C. The living colony formation efficiency was determined by formation of dark-colored formazan dye by reduction of the tetrazolium salt MTS by metabolically active cells. Digital images of the plates were captured by Nikon camera.

**Western blot analysis.** To evaluate the protein expression levels after niclosamide treatment, western blot analysis was performed. The cells were dissolved in a lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic

acid, 1% NP-40, 1 mM EDTA, 5 mM sodium orthovanadate, 25 mM sodium fluoride, 5 mM sodium pyrophosphate decahydrate and 5 mM  $\beta$ -glycerophosphate. Protein concentration was quantified by Bio-Rad protein assay. Fifty micrograms of protein was fractionated via 10 or 12% SDS-PAGE and transferred to Immobilon Transfer Membranes (EMD Millipore), and then the membranes were blocked in 5% skim milk at room temperature for 1 h. Primary antibodies used in the present study included those against  $\beta$ -actin (GTX109639) (dilution 1:10,000; GeneTex, Inc.), poly(ADP-ribose) polymerase (PARP) (9542S),  $\beta$ -catenin (8480S), STAT3 (4904S), phosphor-STAT3 (9145S) (dilution 1:1,000; Cell Signaling Technology, Inc.), cyclin D1 (H295), cyclin E1 (HE-12), cyclin A (H432), and cyclin B (GNS1) (dilution 1:1,000; Santa Cruz Biotechnology, Inc.). The HRP-conjugated secondary antibodies recognizing mouse-IgG (7076) or rabbit-IgG (7074) were purchased from Cell Signaling Technology, Inc. and those recognizing goat-IgG (SC-2020) were purchased from Santa Cruz Biotechnology, Inc. (dilution, ~1:5,000-10,000). The chemiluminescence signal was developed with Immobilon Western Chemiluminescent HRP Substrate (WBKLS0500; Millipore) and visualized and recorded using the BioSpectrum UVP810 Imaging System with VisionWorks software (GE Healthcare Life Sciences).

**Real-time quantitative RT-PCR (RT-qPCR) analysis.** To quantify the endogenous mRNA expression levels, qRT-PCR was performed. Total RNA of cells was isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. One microgram of total RNA was used to synthesize the cDNA using SuperScript III reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) with Oligo-dT primer. Quantitative PCR was performed using Kapa SYBR fast qPCR master mix (Kapa Biosystems, Inc.). The primer sequences for qPCR were as follows: GAPDH forward, 5'-GAAGGTGAAGGTCGGAGT-3' and reverse, 5'-GAAGATGGTGTGGGATTC-3'; p21 forward, 5'-GAAGACCATGTGGACCTGTC-3' and reverse, 5'-TCC TCTGGAGAAGATCAGC-3'; p27 forward, 5'-GTTTCA GACGGTTCCCCAAA-3' and reverse, 5'-CCTTGCTTCATC AAGCAGTG-3'. The qPCR reaction was run in a final volume of 20  $\mu$ l containing 1  $\mu$ l of reverse transcriptase product, 10  $\mu$ l of 2X Kapa SYBR fast qPCR master mix, and 0.6  $\mu$ l of each primer (10  $\mu$ M). The qPCR mixtures were pre-incubated at 95°C for 3 min, followed by 40 cycles at 95°C for 3 sec and 60°C for 30 sec. All qPCR was performed in triplicate using an iQ5 Real-Time PCR detection system (Bio-Rad Laboratories, Inc.). Relative expression was calculated using the  $2^{-\Delta\Delta C_q}$  method (40).

**Annexin V and propidium iodide (PI) double staining.** Following drug treatment, the cells were collected and washed twice with cold PBS, and stained with 5  $\mu$ l of Annexin V-FITC and 10  $\mu$ l of PI (5 g/ml) in 0.5 ml of binding buffer (10 mM HEPES, pH 7.4; 140 mM NaOH; 2.5 mM CaCl<sub>2</sub>) for 15 min at room temperature in the dark. The apoptotic cells were determined using a BD FACSVerser Flow Cytometer (BD Biosciences).

**Cell cycle analysis.** After niclosamide treatments, the cells were collected and fixed with cold 80% ethanol at -20°C for

1 h. The cells were then washed twice with PBS and stained with 0.5 ml PI/RNase at RT for 30 min. The samples were analyzed by BD FACSVerser Flow Cytometer.

**Statistical analysis.** All values were expressed as the mean  $\pm$  standard error. The Student's t-test was used to analyze the results, and a P-value of <0.05 was considered to indicate a statistically significant difference.

## Results

**Niclosamide suppresses the STAT3 signaling pathway and inhibits the cell growth of esophageal cancer cell lines.** Niclosamide, a direct STAT3 inhibitor, had been reported to exhibit the ability to suppress multiple oncogenic signaling transduction pathways. In order to explore the effect of niclosamide on STAT3 and  $\beta$ -catenin signaling pathways of cultured esophageal cancer cells, CE48T, CE81T and BE3 cells were treated with 10  $\mu$ M niclosamide for 24 h, and the total forms of STAT3 and  $\beta$ -catenin and phosphorylated (p)-STAT3 (Y705) were investigated by western blot analysis. As revealed in Fig. 1A, there were different expression levels of  $\beta$ -catenin in these cell lines. The protein levels of endogenous  $\beta$ -catenin could only be detected in CE48T and CE81T cells, but not in BE3 cells. Moreover, niclosamide treatment did not markedly affect the protein level of  $\beta$ -catenin. Conversely, the STAT3 signaling pathway was constitutively active in these cancer cells, and niclosamide treatment markedly suppressed STAT3 (Y705) phosphorylation. Furthermore, to explore the effect of niclosamide on the proliferation of esophageal cancer cells, these cells were treated with niclosamide at different concentrations for 72 h and then cell viability was assessed by MTS assay. As revealed in Fig. 1B, the cell viabilities of these three cancer cell lines were dose-dependently decreased by niclosamide treatment. The IC<sub>50</sub> concentrations of niclosamide were 2.8, 11.3, and 5.1  $\mu$ M for CE48T, CE81T and BE3, respectively. Moreover, a clonogenic assay was conducted to investigate the long-term growth inhibitory effect of niclosamide on esophageal cancer cells. Since CE81T cells did not grow on the soft agar to form a colony, only BE3 and CE48T cells were repeated and long-term treated with a physiological concentration of niclosamide (2.5  $\mu$ M) for 21 days and living colony formation efficiency was determined by formation of dark-colored formazan dye by reduction of the tetrazolium salt MTS by metabolically active cells. As revealed in Fig. 1C, niclosamide completely inhibited colony formation in BE3 and CE48T cells, indicating that niclosamide may be considered as a potential inhibitor for esophageal cancer cell growth.

**Niclosamide treatment induces apoptosis of esophageal cancer cell lines.** To further explore if the decrease in esophageal cancer cell viability was a consequence of niclosamide-induced apoptosis, BE3 cells treated with niclosamide were subjected to western blot analysis of cleaved PARP. As revealed in Fig. 2A and B, the highest level of cleaved PARP product was detected at 10  $\mu$ M niclosamide and 48 h of treatment, indicating that the apoptotic level was positively associated with the niclosamide dose and exposure time in BE3 cells. To further demonstrate the induction of apoptosis

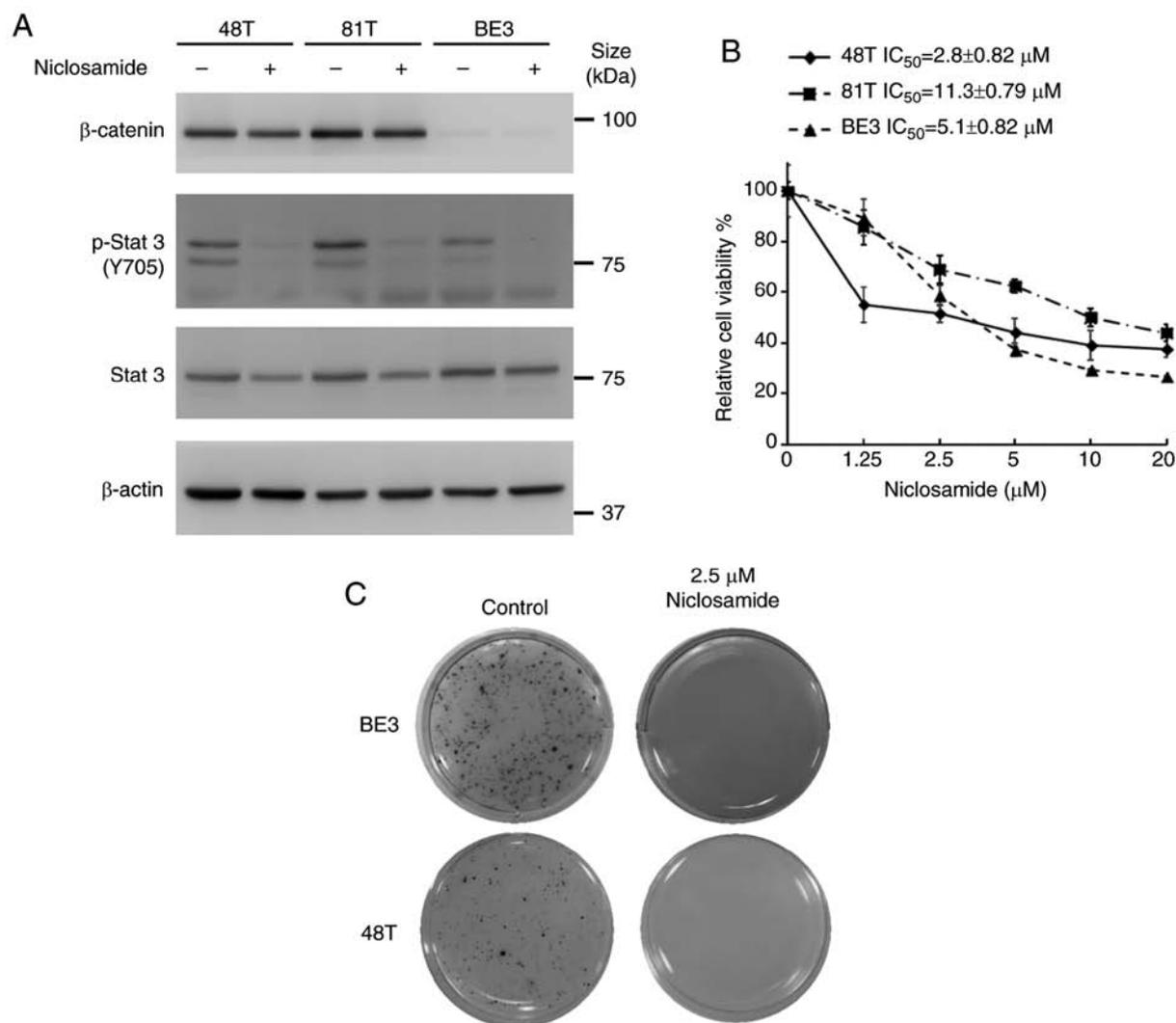


Figure 1. Niclosamide treatment blocks the STAT3 signaling pathway and inhibits the cell growth of human esophageal cancer cell lines. (A) CE48T, CE81T and BE3 cells were treated with  $10 \mu M$  niclosamide for 24 h, and the phosphorylation of Y705 and the total form of STAT3, as well as  $\beta$ -catenin, were investigated by western blot assay using  $\beta$ -actin as a loading control. (B) Cancer cells were seeded in 96-well plates and treated with a concentration series of niclosamide (0, 1.25, 2.5, 5, 10 or  $20 \mu M$ ) for 72 h, and then the relative cell viabilities were determined by MTS assay. Error bars represent the standard deviation. The viability of control cells was designated as 100%, and that of the other groups were expressed as the percent of the control. (C) Colony-forming ability of CE48T and BE3 cells analyzed by clone formation assay. Images of living cell clones indicated by the formation of dark-colored formazan dye by reduction of the tetrazolium salt MTS by metabolically active cells. STAT3, signal transducer and activator of transcription 3; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium.

by niclosamide, CE48T, CE81T and BE3 cells were treated with niclosamide for 48 h, and subjected to Annexin V and PI double-staining assay. The early apoptotic and dead cells were quantified by flow cytometric analysis. As revealed in Fig. 2C-E, niclosamide significantly ( $P<0.05$ ) increased the proportion of early apoptotic cells in the three cell lines, but only significantly increased the proportion of dead cells in CE48T ( $P<0.005$ ) and BE3 ( $P<0.0005$ ) cells. The results indicated that the reduction of cell viabilities of CE48T and BE3 cells was mainly caused by niclosamide-induced cell apoptosis, but was partial to that of CE81T cells.

*Niclosamide treatment causes G1-phase arrest in CE81T cells.* Given that only a small proportion of apoptotic cells were detected in CE81T cells after treatment with  $10 \mu M$  niclosamide for 48 h (Fig. 2D), cell cycle dynamics were analyzed by flow cytometric analysis to investigate why

niclosamide suppressed cell growth of CE81T cells. The cell cycle analysis revealed that after treatment with  $10 \mu M$  niclosamide for 72 h a high proportion of sub-G1 (cell debris) was only detected in CE48T and BE3 cells, while a low proportion of sub-G1 was revealed in CE81T cells. Moreover, an apparent G1 peak was only revealed in CE81T cells, as well as absence of S and G2/M phases (Fig. 3A). In addition, the physiological concentration of niclosamide ( $2.5 \mu M$ ) was also used to conduct cell cycle analysis in CE48T, CE81T and BE3 cells. The proportion of sub-G1 cells was increased in a time-dependent manner in CE48T and BE3 cells after 48 and 72 h treatment. In addition, at 72 h of treatment, the proportion of G1-phase cells was decreased and the cells of the S and G2/M phases were still detectable. In contrast, in CE81T cells, the proportion of sub-G1 cells did not significantly increase, the proportion of G1-phase cells was increased and the proportion of S-phase cells was decreased after 72 h of

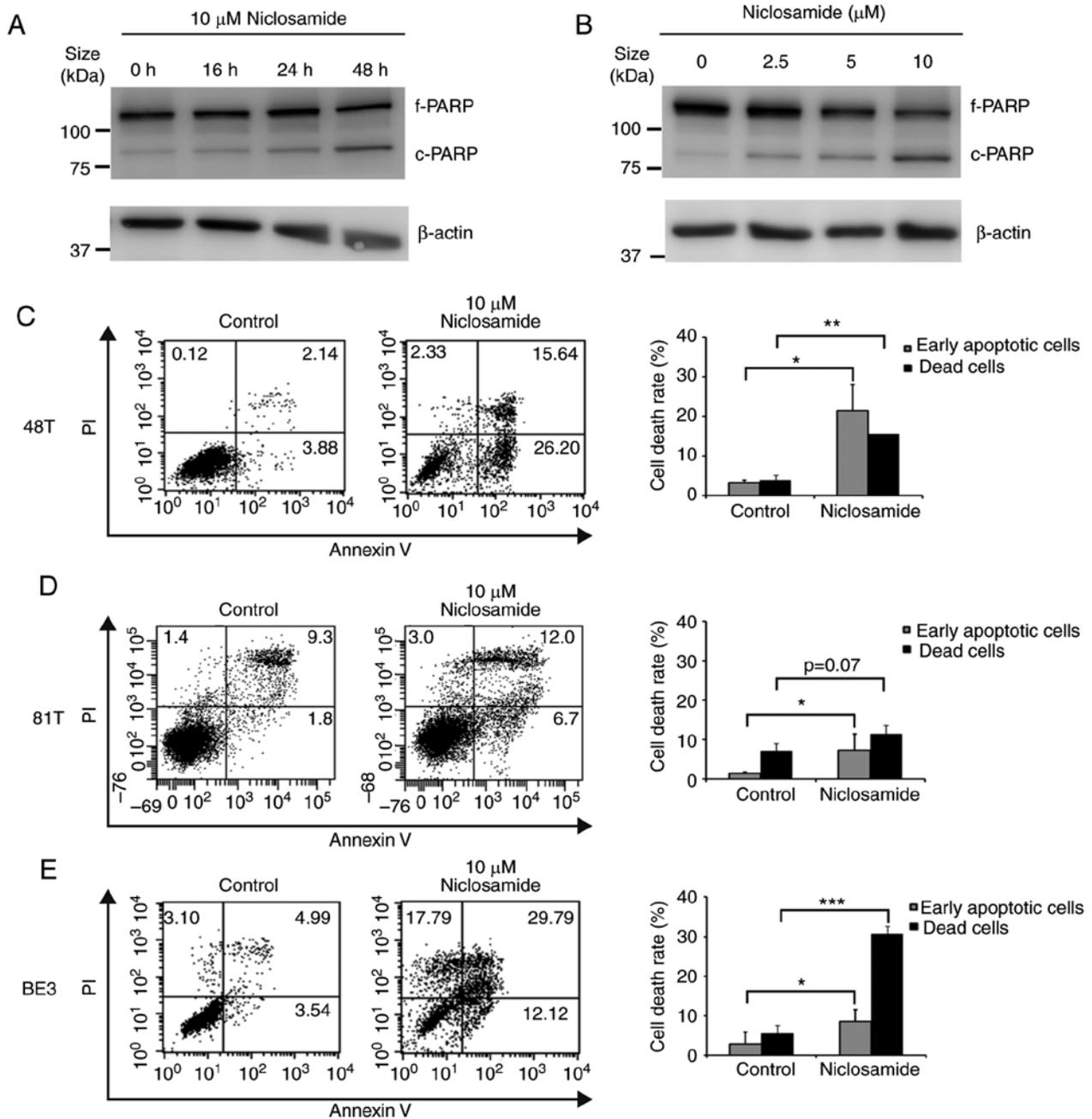


Figure 2. Niclosamide treatment induces apoptosis in esophageal cancer cell lines. (A) BE3 cells were treated with  $10\ \mu\text{M}$  niclosamide, then harvested at different time-points after treatment, or (B) treated with various doses of niclosamide for 48 h. Treated cells were lysed for western blot analysis of PARP. The position of the native PARP (f-PARP) and the cleaved fragment (c-PARP) is indicated. (C) CE48T, (D) CE81T and (E) BE3 cells were treated with  $10\ \mu\text{M}$  niclosamide for 48 h and the apoptosis levels were detected by Annexin V/PI staining. DMSO treatment was used as the control. The proportion of Annexin V<sup>+</sup>/PI<sup>-</sup> and Annexin V<sup>+</sup>/PI<sup>+</sup> cells indicated the early apoptotic and dead cells, respectively. Results were expressed as the means  $\pm$  SD of three independent experiments. \* $P < 0.05$ , \*\* $P < 0.005$ , and \*\*\* $P < 0.0005$ . PARP, poly(ADP-ribose) polymerase.

treatment (Fig. S1). This finding indicated that niclosamide induced G1-phase arrest in CE81T cells. Cyclin is a family of proteins that control the progression of cells through the cell cycle. The protein levels of various cyclins in CE48T, CE81T and BE3 cells were assessed by western blot assay, and it was revealed that all of the expression levels of cyclin D1, E, A and B1 were almost completely lost in CE81T cells treated with niclosamide for 48 h (Fig. 3B). In addition, the expression of cell cycle inhibitor genes, *p21* and *p27*, were also examined in these cells treated with or without  $10\ \mu\text{M}$  niclosamide for 24 h. As revealed in Fig. 3C and D, an  $\sim 11$ -fold increase in *p21*

mRNA expression was detected in niclosamide-treated CE81T cells, as well as an  $\sim 3.5$ -fold increase in niclosamide-treated CE48T and BE3 cells. In contrast, niclosamide treatment did not affect *p27* mRNA expression in these cells.

*Niclosamide co-treatment selectively reduces the dosage of anticancer drugs for achieving  $IC_{50}$  in esophageal cancer cells.* A previous study reported that the combination of niclosamide and anticancer drugs resulted in a synergistic antitumor efficacy against xenografted AML tumor cells (23). 5-FU, cisplatin, and paclitaxel are three frontline chemotherapeutic

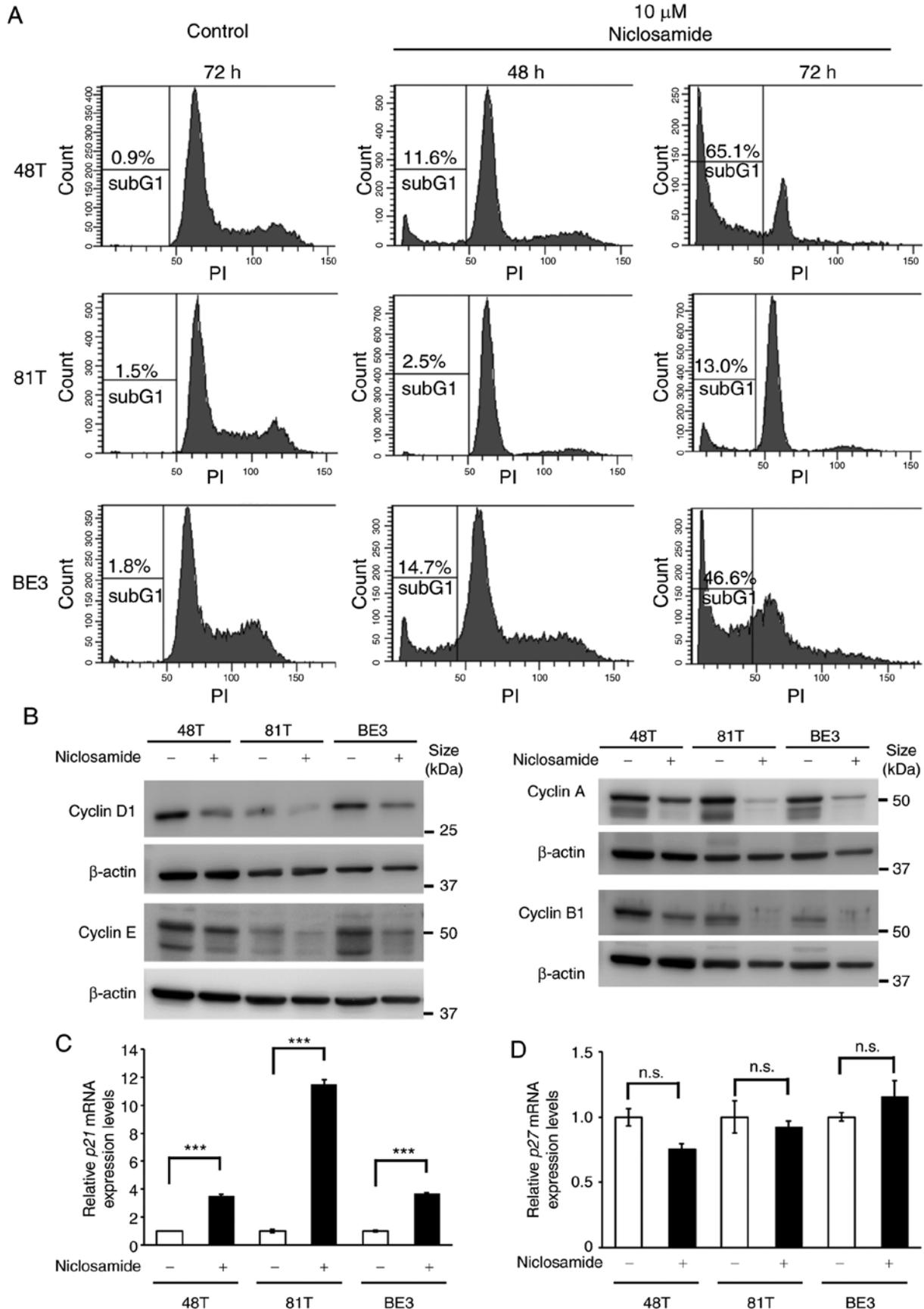


Figure 3. Niclosamide treatment causes CE81T cell cycle arrest. (A) CE48T, CE81T and BE3 cells were treated with 10  $\mu$ M niclosamide for 48 or 72 h. Cells were stained with PI for DNA content and analyzed by flow cytometry. The relative proportion of the sub-G1 cells are indicated. (B) The protein expression levels of cyclin D1, E, A and B1 in CE48T, CE81T and BE3 were determined with western blot assay after cells were treated with 10  $\mu$ M niclosamide for 48 h. Protein loading was normalized based on  $\beta$ -actin. (C and D) These cells were treated with or without 10  $\mu$ M niclosamide for 24 h and total RNA was collected for *p21* and *p27* assessment by RT-qPCR assays. The data were normalized by GAPDH expression and presented as the mean  $\pm$  SEM (n=3). \*\*\*P<0.001; n.s., not significant. PI, propidium iodide.

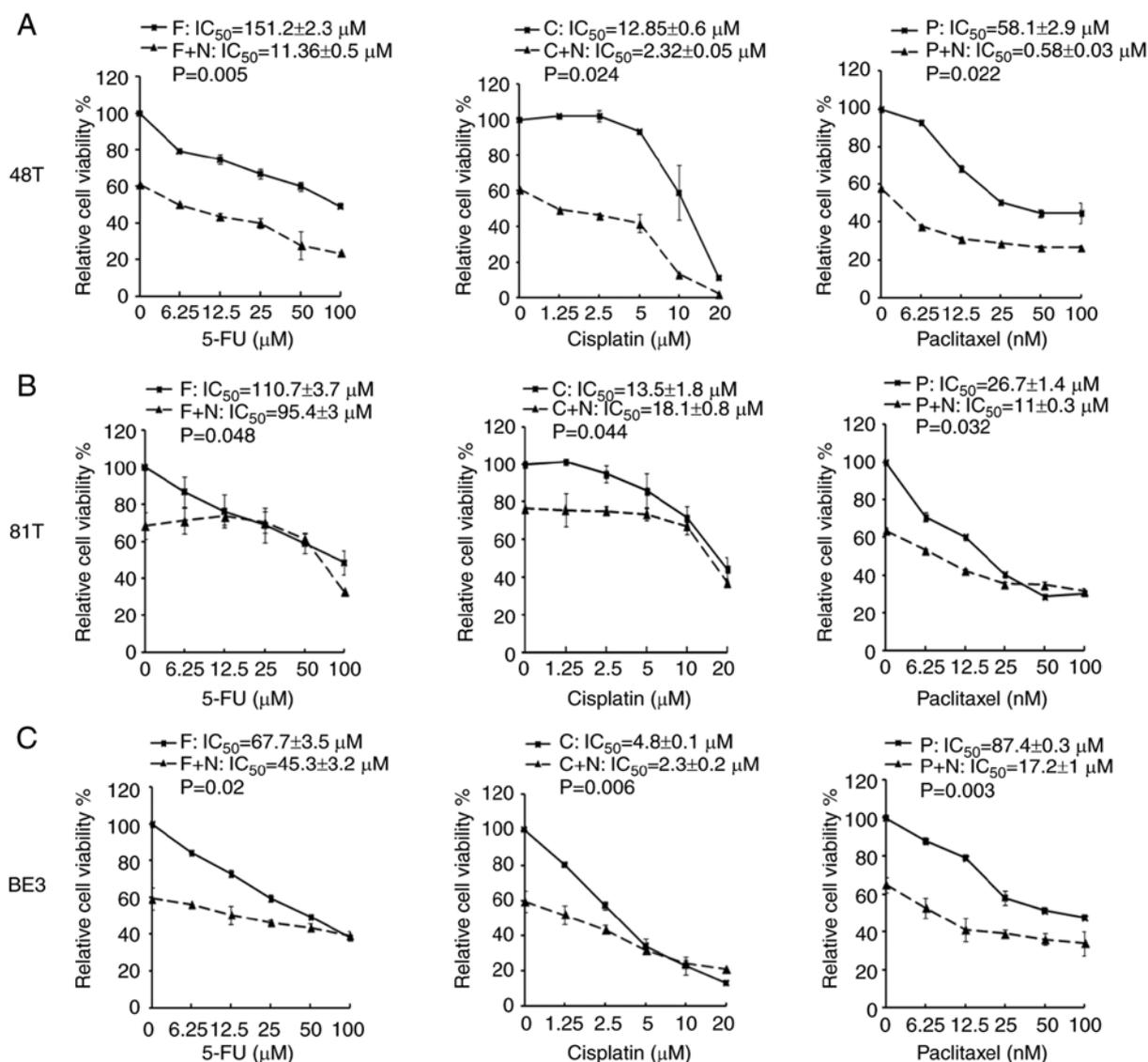


Figure 4. MTS assays of esophageal cancer cells treated with a chemotherapeutic drug alone or in combination with niclosamide. Esophageal cancer cells (A) CE48T, (B) CE81T and (C) BE3 cells were seeded in 96-well plates and treated for 72 h with serial doses of 5-FU, cisplatin, paclitaxel, or in combination with 2.5  $\mu\text{M}$  of niclosamide. Cell viability was then analyzed by MTS assay. F, C, and P indicate the treatment of 5-FU, cisplatin, or paclitaxel, respectively. F+N, C+N, and P+N indicate the combination treatment of niclosamide with 5-FU, cisplatin, or paclitaxel, respectively. 5-FU, 5-fluorouracil.

drugs currently used in clinical esophageal therapy (41-43). Therefore, they were used in the present experiment to explore whether the treatment of these anticancer drugs combined with niclosamide could have an additive benefit of antitumor efficacy for esophageal cancer cells. Niclosamide appears to be minimally absorbed from the gastrointestinal tract and a pharmacokinetics study revealed that the oral dosage of niclosamide for adults in cytotoxic treatment was 2 g as a single dose, leading to maximal serum concentration of 0.76 to 18.35  $\mu\text{M}$  (44). In the present study, the esophageal cancer cell lines were treated with the combination of 2.5  $\mu\text{M}$  niclosamide plus different chemotherapeutic drugs for 72 h, and cell viability was assessed by MTS assay. In CE48T and BE3 cells, the IC<sub>50</sub> values of 5-FU, cisplatin, and paclitaxel were reduced in the combination groups of niclosamide, when compared to the anticancer drug administered alone (Fig. 4A and C). In CE81T cells, the niclosamide combination only reduced the IC<sub>50</sub> values of 5-FU and paclitaxel as

compared with anticancer drug treatment alone (Fig. 4B). To further validate the benefit of combination treatment with niclosamide, the proportion of apoptotic cells (Annexin V<sup>+</sup> cells) induced by the anticancer drug alone, or in combination with niclosamide was investigated by flow cytometric analysis. As revealed in Fig. 5, the treatment of 2.5  $\mu\text{M}$  niclosamide for 48 h induced 18.46 ± 3.06% of apoptotic cells in CE48T cells, and the combination of niclosamide and 5-FU (50  $\mu\text{M}$ ) caused a higher proportion of apoptotic cells than 5-FU treatment alone (26.14 ± 5.89% vs. 8.62 ± 1.47%; P < 0.05). Moreover, the combination of niclosamide and cisplatin (10  $\mu\text{M}$ ) resulted in a higher proportion of apoptotic cells than cisplatin treatment alone (56.68 ± 1.82% vs. 25.57 ± 8.81%; P < 0.05), as well as the combination of niclosamide and paclitaxel (10 nM) which induced a higher proportion of apoptotic cells than paclitaxel treatment alone (28.69 ± 1.75% vs. 12.54 ± 1.12%; P < 0.005). In CE81T cells, there was no statistically significant difference detected in the proportion of apoptotic cells between

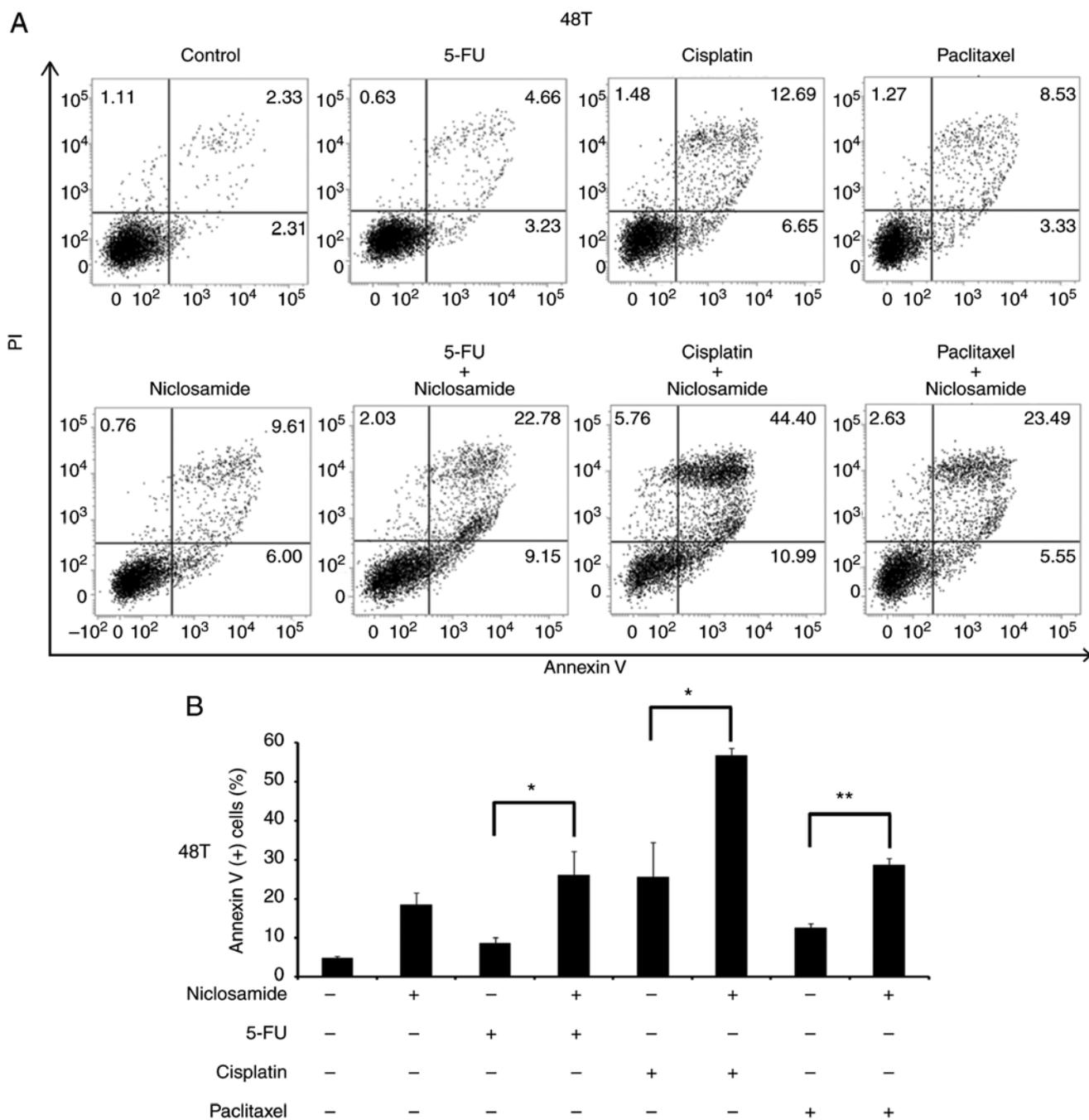


Figure 5. Cell apoptosis assay of CE48T cells using Annexin V/PI double-staining method. (A) CE48T cells were treated with niclosamide (2.5  $\mu$ M), 5-FU (50  $\mu$ M), cisplatin (10  $\mu$ M), paclitaxel (10 nM) alone, or in combination with niclosamide and each of these chemotherapeutic drugs for 48 h. The apoptotic levels were detected by Annexin V/PI staining and analyzed by flow cytometric assay. DMSO treatment was used as the control. The proportion of Annexin V (+) cells indicate the stage of apoptosis. (B) The percentage of Annexin V (+) cells were expressed as the means  $\pm$  SD of three independent experiments. \* $P$ <0.05 and \*\* $P$ <0.005. 5-FU, 5-fluorouracil.

treatment with these three chemotherapeutic agents alone and the combination of niclosamide with 5-FU, cisplatin, or paclitaxel (as revealed in Fig. 6). In BE3 cells, the combination of niclosamide and paclitaxel induced a higher proportion of apoptotic cells than paclitaxel treatment alone ( $39.88 \pm 6.83\%$  vs.  $21.26 \pm 3.26\%$ ;  $P$ <0.05) (as revealed in Fig. 7). The results of the cell apoptosis assays were similar with the findings of the cell viability assays, indicating that niclosamide selectively reduced the dosage requirement of anticancer drug for obtaining the  $IC_{50}$  in esophageal cancer cells.

## Discussion

The current chemotherapeutic strategy is inevitably accompanied by a variety of dose-dependent and sometimes lethal adverse effects. Thus, it is worth exploring cost-effective drug repurposing and combinations to ameliorate unpleasant adverse effects following traditional chemotherapy. Niclosamide is an FDA-approved anthelmintic drug which exerts antineoplastic activity. The antineoplastic efficacy of niclosamide in numerous human cancer studies (8-35) has been demonstrated.

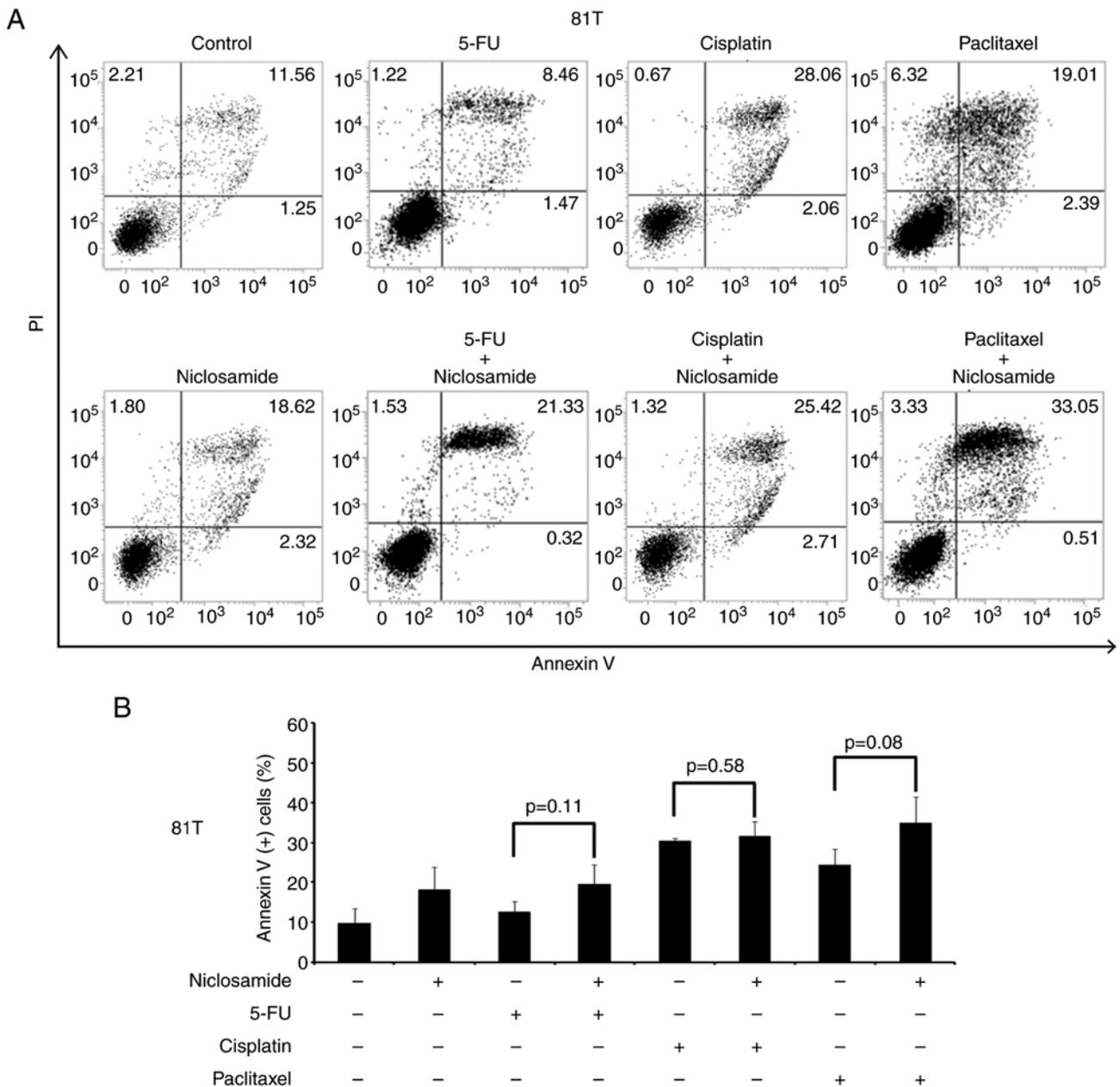


Figure 6. Cell apoptosis assay of CE81T cells using Annexin V/PI double-staining method. (A) CE81T cells were treated with niclosamide (2.5  $\mu$ M), 5-FU (50  $\mu$ M), cisplatin (10  $\mu$ M), paclitaxel (10 nM) alone, or in combination with niclosamide and each of these chemotherapeutic drugs for 48 h. The apoptotic levels were detected by Annexin V/PI staining and analyzed by flow cytometric assay. DMSO treatment was used as the control. The proportion of Annexin V (+) cells indicate the stage of apoptosis. (B) The percentage of Annexin V (+) cells were expressed as the means  $\pm$  SD of three independent experiments. 5-FU, 5-fluorouracil.

However, whether niclosamide is active against esophageal cancer is unknown. Herein, the present study for the first time demonstrated that niclosamide exhibited antineoplastic effects in esophageal cancer cell lines.

Niclosamide has been identified as a direct STAT3 inhibitor through interaction with the DNA-binding domain (37). In addition to its role in STAT3 inhibition, niclosamide has also been revealed to concurrently inhibit multiple intracellular signaling pathways, including the Wnt, Notch 1, mTOR and NF- $\kappa$ B signaling cascades (10,45). In many solid and hematological cancers, STAT3 is constitutively active, promoting tumor survival and progression. For example, a previous study by Huang *et al* reported that positive p-STAT3 expression

was observed in 47.7% of patients with esophageal squamous cell carcinoma. Patients with p-STAT3 positive disease had significantly worse clinical outcomes in terms of median progression-free survival (5.0 vs. 6.9 months,  $P < 0.001$ ) and median overall survival (8.9 vs. 9.9 months,  $P = 0.026$ ) (46). In addition, Wang *et al* reported that constitutively activated STAT3 in normal human esophageal epithelium cells (HET-1A) promoted proliferation and decreased apoptosis. Moreover, STAT3 activated HET-1A cells to form tumors *in vivo* (47), suggesting that aberrant STAT3 expression plays a crucial role in esophageal carcinogenesis. Conversely, STAT3 knockdown significantly reduced cell proliferation of OE21 (ESCC) and OE33 (EAC) cells, and a reduced STAT3 level was revealed

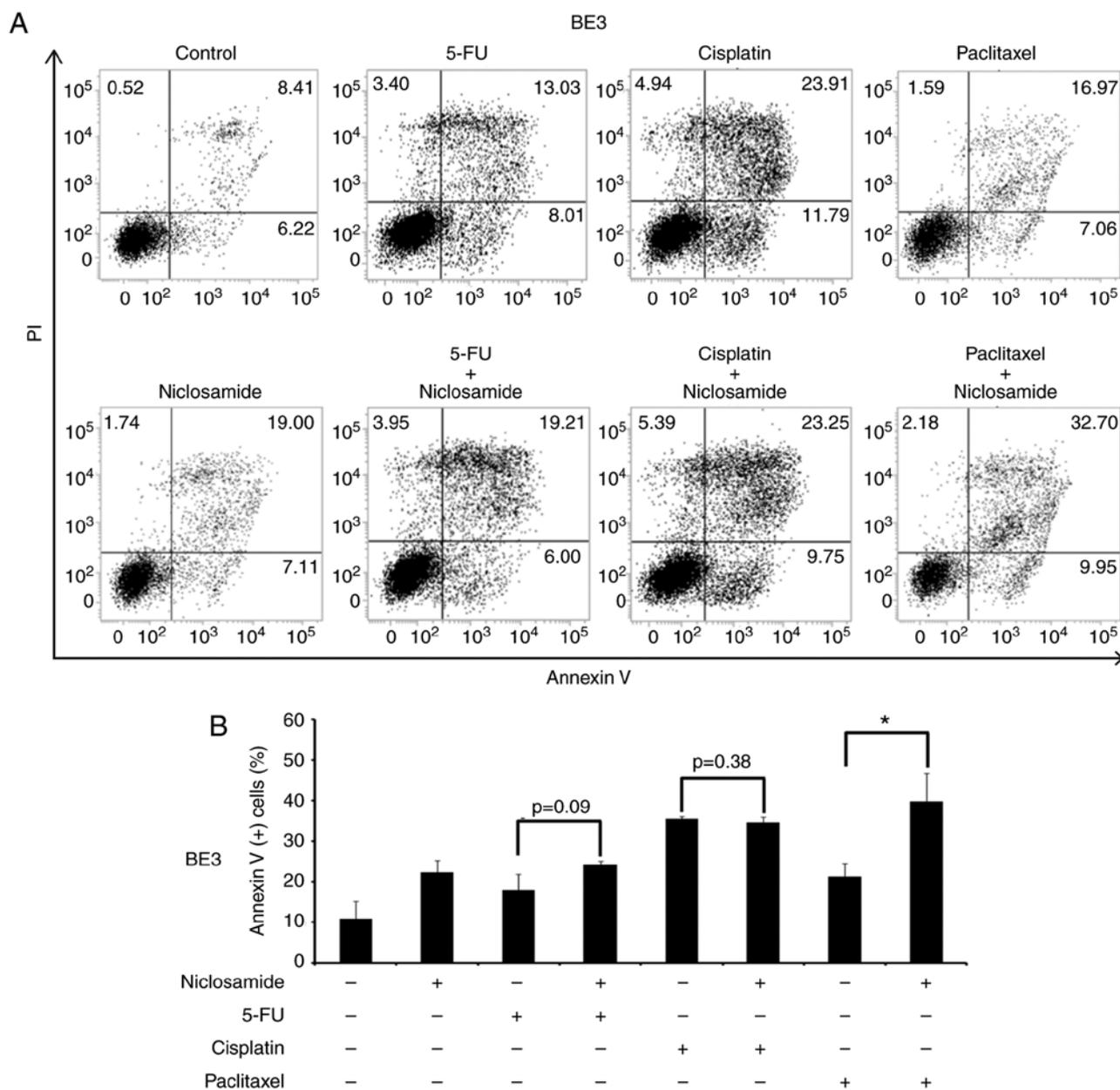


Figure 7. Cell apoptosis assay of BE3 cells using Annexin V/PI double-staining method. (A) BE3 cells were treated with niclosamide (2.5  $\mu$ M), 5-FU (50  $\mu$ M), cisplatin (10  $\mu$ M), paclitaxel (10 nM) alone, or in combination with niclosamide and each of these chemotherapeutic drugs for 48 h. The apoptotic levels were detected by Annexin V/PI staining and analyzed by flow cytometric assay. DMSO treatment was used as the control. The proportion of Annexin V (+) cells indicated the stage of apoptosis. (B) The percentage of Annexin V (+) cells were expressed as the means  $\pm$  SD of three independent experiments. \* $P$ <0.05. 5-FU, 5-fluorouracil.

to be associated with significant downregulation of cell cycle genes in both cell lines (48), indicating that STAT3 plays a pivotal role in cell proliferation of esophageal cancer cells. Through western blot analysis, it was revealed that the STAT3 signaling pathway, but not the Wnt pathway, was constitutively active in the three esophageal cancer cell lines. In addition, niclosamide treatment did not only markedly suppress the phosphorylation of Y705 of STAT3, but also slightly decreased the total protein levels of STAT3. Similar results were also obtained by Yoshikawa *et al* in a niclosamide-treated ovarian clear cell line (KK) (49). However, the mechanism resulting in the decrease of STAT3 total protein form by niclosamide treatment is still unclear. The  $IC_{50}$  values of niclosamide were 2.8, 11.3, and 5.1  $\mu$ M for CE48T, CE81T and BE3 cells,

respectively. Notably, ESCC CE81T cells were more resistant than ESCC CE48T cells to niclosamide. Similar resistant responses were revealed in a previous study in which CE81T cells were resistant to photofrin-induced inhibition of EGFR as well as to photofrin-mediated dark toxicity, compared with CE48T cells (39). Since niclosamide is minimally absorbed from the gastrointestinal tract, 2.5  $\mu$ M niclosamide was used, a concentration less than the  $IC_{50}$  value, to investigate the long-term growth inhibitory effect of niclosamide in esophageal cancer cells by colony-forming assay. Niclosamide completely inhibited colony formation in BE3 and CE48T cells after 21 days of treatment, indicating that niclosamide may be considered as a potential inhibitor of esophageal cancer cell growth, as demonstrated in other types of cancer cells.

According to the results of western blot analysis of PARP and the Annexin V/PI staining assay, it was demonstrated that the inhibitory effect of niclosamide on cell growth in esophageal adenocarcinoma cell line BE3 was caused by niclosamide-induced apoptosis. By contrast, in esophageal squamous cell carcinoma cell lines, niclosamide significantly ( $P < 0.05$ ) increased the proportion of early apoptotic cells in both CE48T and CE81T cells, however only significantly increased the proportion of dead cells in CE48T ( $P < 0.005$ ), but not in CE81T cells. After 48 h of treatment of niclosamide, only a small proportion of Annexin V-positive cells were detected in CE81T, compared with CE48T and BE3 cells. The detailed mechanism of the different response of CE81T cells to niclosamide compared to CE48T and BE3 cells remains unclear and further investigation is required.

Previous studies in oral squamous cell carcinoma (26), adrenocortical carcinoma (8) and head and neck cancer (18) and osteosarcoma (25) revealed that niclosamide could induce G1-phase arrest of the cell cycle. Through flow cytometric analysis using DNA dye PI staining, it was revealed that a high proportion of sub-G1 cells were detected in CE48T and BE3 cells after 10  $\mu\text{M}$  niclosamide treatment for 72 h, while only a small proportion of sub-G1 was detected in CE81T cells. Moreover, an evident G1 peak was revealed in CE81T cells. Similar experiments were also performed using the physiological concentration of niclosamide (2.5  $\mu\text{M}$ ) and the results were consistent with the high dose treatment. In addition, the protein levels of the various cyclins were explored by western blot assay, and it was revealed that the protein levels of cyclin D1, E, A and B1 almost completely disappeared in CE81T cells after niclosamide treatment for 48 h. As G1-phase arrest, cell cycle checkpoint proteins, such as p21 and p27, may mediate assembly and activation of Cdk4(6)-cyclin D in the cytoplasm, as well as inhibiting their activity in the nucleus. Ectopic induction of p21 or p27 expression completely arrested cells at the G1 phase of the cell cycle by p27 and at G1 and G2 phases by p21 (50). Therefore, in the present study, it was explored whether p21, p27 or both were involved in niclosamide-induced G1-phase arrest in CE81T cells. As revealed, niclosamide after 24 h of treatment significantly increased *p21* mRNA expression in CE81T cells, but not *p27* mRNA. These findings demonstrated that niclosamide induced G1-phase arrest in ESCC CE81T cells, however, the detailed mechanism is not clear yet.

Niclosamide may be beneficial not only as a monotherapy but also in combination therapy. Niclosamide has been demonstrated to increase the sensitivity of cancer cells for radiation therapy in lung cancer (51), nasopharyngeal cancer (24) and breast cancer (9). Furthermore, niclosamide was also revealed to exert a synergistic effect with numerous cancer drugs in human cancer cells. Compared with cancer drug treatment alone, the combination treatment of niclosamide with bicalutamide, cisplatin, paclitaxel, carboplatin, erlotinib, dasatinib, or sorafenib exhibited an improved antineoplastic efficacy in prostate (32), lung (52), cervical (14), ovarian (30), colon (53), chronic myeloid leukemia (22) and renal cancer (34), respectively. To date, the frontline chemotherapeutic agents for advanced esophageal cancer cells are 5-FU, cisplatin, and paclitaxel. In the present study, the antineoplastic efficacy of combination treatment of niclosamide (2.5  $\mu\text{M}$ ) plus 5-FU, cisplatin, or paclitaxel was assessed in esophageal cancer cells, and it was

revealed that niclosamide co-treatment selectively reduced the dosage of anticancer drugs for obtaining the  $\text{IC}_{50}$ . In CE48T and BE3 cells, the  $\text{IC}_{50}$  values of 5-FU, cisplatin, or paclitaxel were reduced in the combination groups with niclosamide, when compared to the anticancer drug administered alone. In CE81T cells, niclosamide combination treatment only reduced the  $\text{IC}_{50}$  values of 5-FU and paclitaxel as compared with the anticancer drug treatments alone. The beneficial effects of niclosamide combination on inhibition of cancer cell proliferation were further validated in the cell apoptosis assays. These findings indicated that niclosamide may be used as a repurposing drug to reduce the dosage requirement of anticancer drug for achieving the  $\text{IC}_{50}$  in esophageal cancer cells.

Sustained inhibition of tumor colony formation by long-term niclosamide treatment with potentially achievable serum concentration is indicative of a novel maintenance strategy for esophageal cancers adjunctive to current curatively local treatments or palliative chemotherapy with limited choices and survival benefits but well-known side effects. Due to approved indication of niclosamide as an antiparasitic, there is little clinical data with regard to the adverse effects of prolonged niclosamide treatment. Andrews *et al* reviewed associated results in laboratory animals (including mice, rats and dogs) and reported few cumulative or long-term toxicities across species. They mentioned that there was no indication that a higher incidence of tumors occurred. From all long-term experiments it is evident that the closely-related substances niclosamide ethanolamine salt and niclosamide are not high-risk substances (44). To date, at least three clinical trials for niclosamide in prostate and colon cancers are registered, due to cumulative encouraging laboratory results. There is no reported early termination due to unexpected/unacceptable adverse effects. It is anticipated that if the combination of niclosamide and chemotherapeutic drugs was used clinically it would offer two important advantages: A reduction in the dose requirement of chemotherapeutic drugs, and amelioration of the side effects caused by aggressive treatment with high doses of chemotherapy.

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#### Availability of data and materials

All data used during the current study are available from the corresponding author on reasonable request.

#### Authors' contributions

MCL and BRL conceived and designed the experiments. MCL and YJH performed the experiments. MCL, BRL and YKC collected and analyzed the data, and interpreted the findings. MCL wrote the manuscript. BRL and YKC edited the manuscript. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

#### Ethics approval and consent to participate

Not applicable.

#### Patient consent for publication

Not applicable.

#### Competing interests

There authors declare that they have no competing interests.

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