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EnDuo, a novel derivative of Endostar, inhibits the migration of colon cancer cells, suppresses matrix metalloproteinase-2/9 expression and impedes AKT/ERK activation

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ABSTRACT

Background/aims: Colon cancer remains a life-threating disease with increasing morbidity and mortality worldwide despite the advancement in modern medical treatment. Therefore, novel and effective anti-colon cancers drugs are urgently needed. In this study, we investigated the anti-metastatic property EnDuo, a modified version of Endostar, and the underlying mechanisms.

Methods: Colon cancer cells were treated with different concentrations of EnDuo (50 μg/mL, 100 μg/mL, 200 μg/mL), and Endostar (100 μg/mL) as positive control. Cell Counting Kit-8 assay was performed to test the effect of EnDuo on cell viability. A scratch wound assay and transwell assay were employed to evaluate the relocation and motility of malignant colon cells following treatment with EnDuo. Western blot analysis was used to determine inhibitory effects of EnDuo by detecting the phosphorylation level of AKT and ERK proteins, and the expression of MMP-2 and MMP-9 proteins.

Results: Our results showed that EnDuo impedes the migration of colon cancer cells in a dose-dependent manner. At the molecular level, EnDuo induced a significant reduction in the phosphorylation of AKT and ERK proteins, and inhibited the expression of MMP-2 and MMP-9 proteins.

Conclusions: Collectively, these results demonstrate that EnDuo exhibits a comparable anti-metastatic effect by suppressing the migration of colon cancer cells. Possibly, EnDuo interrupts the PI3K/AKT/ERK signaling pathway to arrest cell migration. Our study provides a novel insight to the potential clinical applications of EnDuo against colon cancers in the future.

1. Introduction

Globally, colorectal cancer (CRC) is rated the second most common cancer diagnosed in women and the third frequently diagnosed malignancy in men [1]. The global incidence of CRC is on a positive trajectory, with the number of new cases rising from 1.2 million in 2008 [2] to over 1.8 million in the year 2018, and approximately a million deaths expected [3]. Early diagnosis of the disease has been associated with successful treatment outcome including surgical intervention. However, advanced stage of CRC is often difficult to treat, and characterized by a high chance of relapse [4]. Neoadjuvant and adjuvant chemotherapeutic agents are commonly used before and post-surgery respectively. 5-Fluorouracil (5-FU), capecitabine (xeloda), irinotecan (camptosar), oxaliplatin (eloxatin), and trifluridine/tipiracil (Lonsurf) are commonly used chemotherapeutic agents in the treatment of CRC. These agents are however associated with various side effects including drug resistance and toxicity [5,6]. The adverse effects associated with the various chemotherapeutic agents demand the need to find new ways to improve existing drugs while hunting for better alternatives, especially chemo-preventive drugs [7]. Thus, there is a pressing necessity for new chemo-preventive or chemotherapeutic agents which are safer to the user and provides optimum protection or curative effect against CRC.

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Recent studies have derived bioactive compounds from natural products, and these compounds have demonstrated efficacy as chemo-preventive drugs or chemotherapeutic drugs. Additionally, studies have shown that some of these bioactive compounds enhance the efficacy of some chemotherapeutic agents.

One of the major characteristics associated with cancers in general and is responsible for most deaths, is their potential to assume a state of metastasis [8,9]. This is when cancer cells inside the primary tumor separate from neighboring cells and invade the basal membrane. Subsequently, regional invasion ensues via epithelial-mesenchymal transition (EMT) [10–12]. Hence, any agent which can curb the growth repression, invasion and migration of cancer cells in the colon with minimal or no side effect could be essential in the treatment CRC, and possibly other cancers.

Endostatin, a 20-kDa C-terminal part of collagen XVIII, is endogenously produced, and it has been shown to be among the most effective agents of anti-angiogenesis in experimental models [13,14]. Endostatin was discovered in a murine hemangioendothelioma cell line in the year 1997, and its discovery has led to an increased understanding in angiogenesis. Recombinant endostatin effectively blocks angiogenesis and suppresses metastasis and primary tumors in animal models without any obvious side effects [15–17]. Even though endostatin has been approved by the US food and drug administration (FDA), its clinical efficacy has not been as effective as anticipated. A new recombinant human endostatin, called Endostar, with extra nine amino acid (MGGSHHHHH) sequence has been shown to be at least twice as effective as endostatin in tumor models in animal [18]. In the 2005, Endostar was accepted by the State Food and Drug Administration of China for the treatment of non-small-cell lung cancer. Nevertheless, the molecular mechanisms of endostatin and endostar in the suppression of tumor growth are still not clear [17].

Matrix Metalloproteinases (MMPs) are a family of transmembrane proteolytic zinc-containing enzymes, and can collectively digest approximate all extracellular matrix and basal membrane components [19]. This characteristic of MMPs contributes substantially to promoting tumor metastasis and angiogenesis [20,21]. Specifically, the gelatinases (MMP-2 and MMP-9) activities correlate with the invasive capability of cancers of the neck, head squamous cell carcinomas [22,23], and in breast malignancy [24]. MMPs are excreted as inactive zymogens with a pro-peptide domain, and they only become biologically active upon the cleavage of the pro-peptide. Actuation of MMPs can be reached *in vitro* by aminophenylmercuric acetic acid derivative (APMA) [25], or *in vivo* in a system with various proteases such as tumor-related trypsinogen-2 (TAT-2) [26–28]. Kim et all [28] have indicated that endostatin substantially decreases intrusion of endothelial cells and tumor cells into a reconstituted basal membrane by suppressing the synergistic activities of MT1-MMP/MMP-2 and the activation of proMMP-2 [29,30]. It has been proven that certain MMPs (such as MMP-3, MMP-9, MMP-12, MMP-13, MMP-20, MMP-2 and MMP-14) can produce endostatin containing peptides from human XVIII collagen type, with contrasting molecular weight (20–30 kDa) [31]. These peptides hinder the migration and proliferation of human umbilical vein endothelial cells [32].

The enhanced anti-cancer activity demonstrated following modification of endostatin to endostar suggests alternative modifications of endostatin could result in a more enhanced therapeutic efficacy against various cancers. It has been reported that the folded structure of endostatin can influence the antitumor activity of insoluble endostatin derived from *Escherichia coli* [33]. Shorter versions of endostatin were therefore synthesized, and the antitumor properties of the NH2-terminal 27-amino acid endostatin peptide were found to be comparable to full-length endostatin [34]. Similarly, different peptides consisting of different amino acids lengths have been developed and investigated for their ability to inhibit tumor growth [35]. Against this background, 'EnDuo', a novel derivative of Endostar, was investigated for its therapeutic potency *in vitro* using selected colon cancer cell lines. The evidence from this study suggests that EnDuo exhibits inhibitory effects on the proliferation and migration of colon cancer cells. Mechanistically, EnDuo interrupts the PI3K/AKT and ERK/MAPK pathways, and restricts the expression of MMP- 2/9 *in vitro*.

2. Materials and methods

2.1. Cell lines

The following three human colon cancer cell lines were used in this study: (1) The SW 620 colorectal adenocarcinoma cells. These cells synthesize small amount of carcinoembryogenic antigens and are highly tumorigenic in nude mice. The established cell line consists of small, spherical and bipolar cells resembling microvilli; (2) HT 29 colorectal adenocarcinoma, is a cell line with epithelial morphology. In addition to being used as a xenograft tumor model for colorectal cancer, the HT-29 cell line is also used as an *in-vitro* model to study absorption, transport, and secretion by intestinal cells, and (3) HCT 116 colorectal carcinoma cells are adherent with an epithelial morphology. Following implant into immunocompromised mice, the cells form primary tumors and metastasize. All the cells were acquired from the Cell Bank of the Chinese Academy of Science (Shanghai, China). The cells were grown in RPMI-1640 medium (Model Number 61870150. Lot: 1930752, GIBCO, Grand Island, NY, USA) complemented with 10 % fetal bovine serum (FBS) (Catalog Number 76237-676, Hyclone, Logan, UT, USA), and 100 U/mL penicillin, and incubated in a 5% CO_2 humidified atmosphere at 37 °C.

2.2. Reagents

Purified form of endostar was purchased from Simcere Pharmaceutical Research Co., Ltd. (Shandong, China). Stocks of synthetic EnDuo (1 μg/mL) were synthesized by Harbin Medical University (Harbin, China) and were delivered in sterile distilled water, and stored at −20 °C. Phenylmethylsulfonyl fluoride (PMSF) (CAS Number 329-98-6) was obtained from Sigma-Aldrich. Bicinchoninic acid (BCA) protein assay kit and RIPA lysis buffer were bought from Beyotime Institute of Technology (Shanghai, China). The primary antibodies used in this study include p-AKT (Ser473) rabbit mAb (1:1,000; cat. no. 4060), AKT rabbit mAb (1:1,000; cat. no. 4691), phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) rabbit mAb (1:1,000; cat. no. 4370), p44/42 MAPK (ERK1/2) rabbit mAb (1:1,000; cat. no. 4695), MMP-2 rabbit mAb (1:1,000; cat. no. 87809), MMP-9 rabbit mAb (1:1,000; cat. no. 13667), GAPDH rabbit mAb (1:1,000; cat. no. 2118) and Horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:1,000; cat. no. 7074) were all acquired from Cell Signaling Technology, Inc. (Danvers, MA, USA).

2.3. Cell counting Kit-8 assay

Cell Counting Kit-8 (CCK-8) assay was performed to test the effect of EnDuo on the viability of the cancer cells. Firstly, 5×10^3 cells in 100 μl per well were seeded in 96-well flat-bottomed plates for 24 h to acclimatize. The cells were then treated with varied concentrations of the drug and incubated for 48 h. When the treatment duration was over, 10 μl of CCK-8 was added to each well, and the plate incubated for additional 4 h in a humidified CO_2 incubator at 37 °C. The absorbance of each well was measured with a microplate reader at 450 nm. The cells viability was estimated by the formular: (Mean absorbance of control – Mean absorbance of treatment)/Mean absorbance of control.

2.4. Wound healing assay

The cells were cultured in a 6-well plate with complete medium until 90 % confluence growth. The confluent cell monolayer was wounded by streaking across it using a sterile 200 μL pipette tip. After that the plate was washed three times with PBS to remove cell debris. Afterwards,

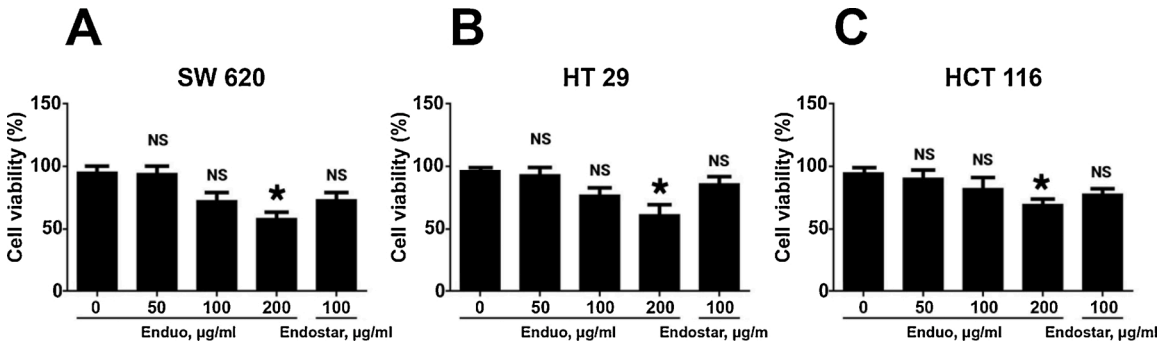

Fig. 1. Cell viability of colon cancer cells assessed by CCK-8 assay following treatment with EnDuo and Endostar. Viability of SW 620 (A), HT 29 cells (B) and HCT 116 (C) after treatment with EnDuo and Endostar for 24 h. The value for each concentration tested represents the mean ± SD of three independent experiments with six replicates. *P < 0.01 vs control.

initial image of the wound were captured and the cells incubated with medium containing EnDuo (0−200 µg/mL) or Endostar (100 µg/mL) for 72 h. The degree of wound closure was measured microscopically at different time points. The migration rate was presented as the ratio of the migrated distance of the cells in the experimental group to that of the control group. Three autonomous experiments were conducted in triplicate and the results presented as mean ± SD.

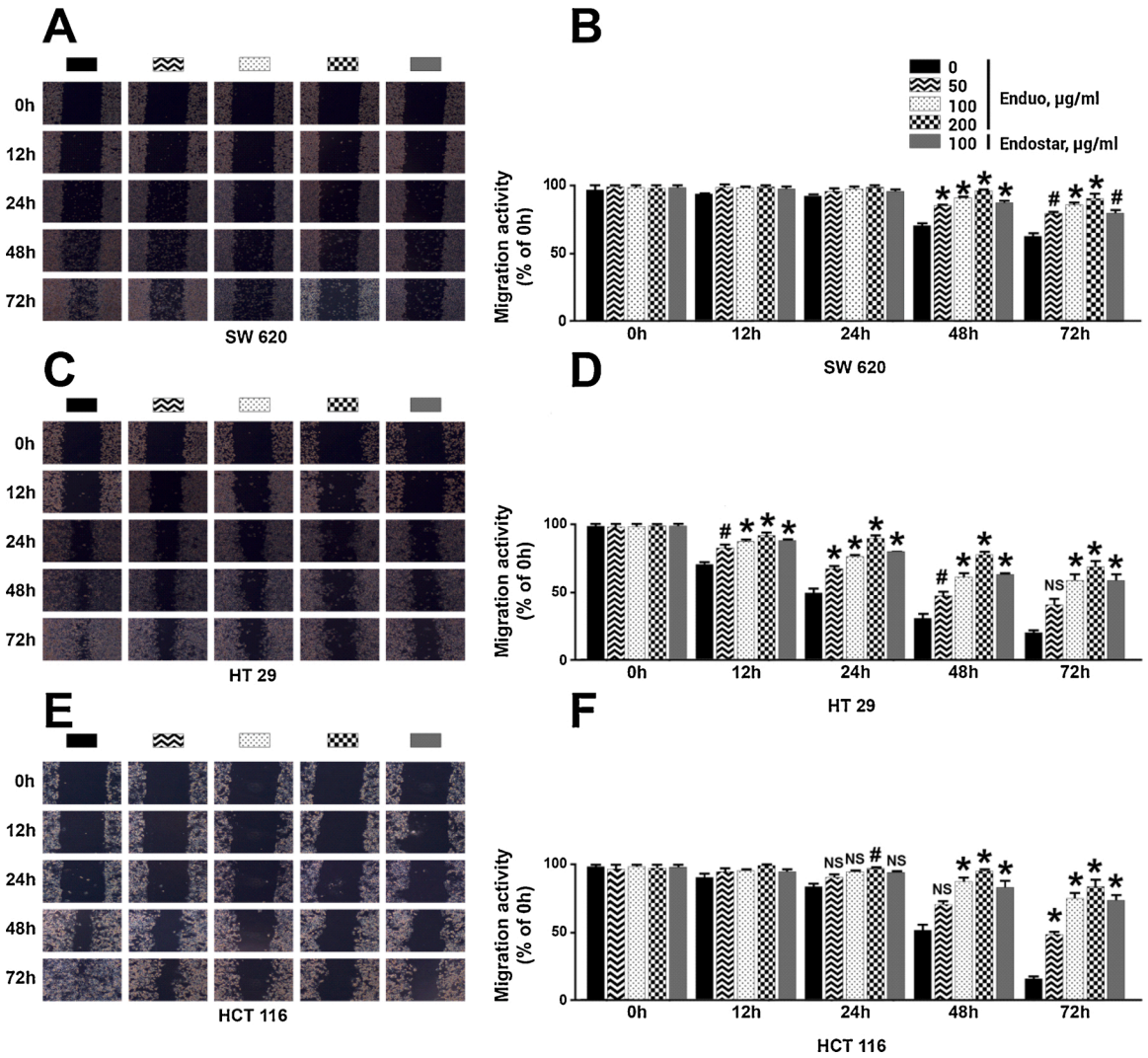

Fig. 2. Scratch wound assay assessment of the inhibitory effect of EnDuo on colon cancer migration following treatment with EnDuo and Endostar (0-200 µg/mL). Respective representative images showing the wound-induction and quantification of wound closured restriction compared to the control for (A) 620, (C) HT 29 and (E) HCT 116 colon cancer cells (Scale bar 200 mm). The data are presented as the mean ± SD of three independent experiments in triplicate. #P < 0.05 vs. the control, *P < 0.01 vs. the control.

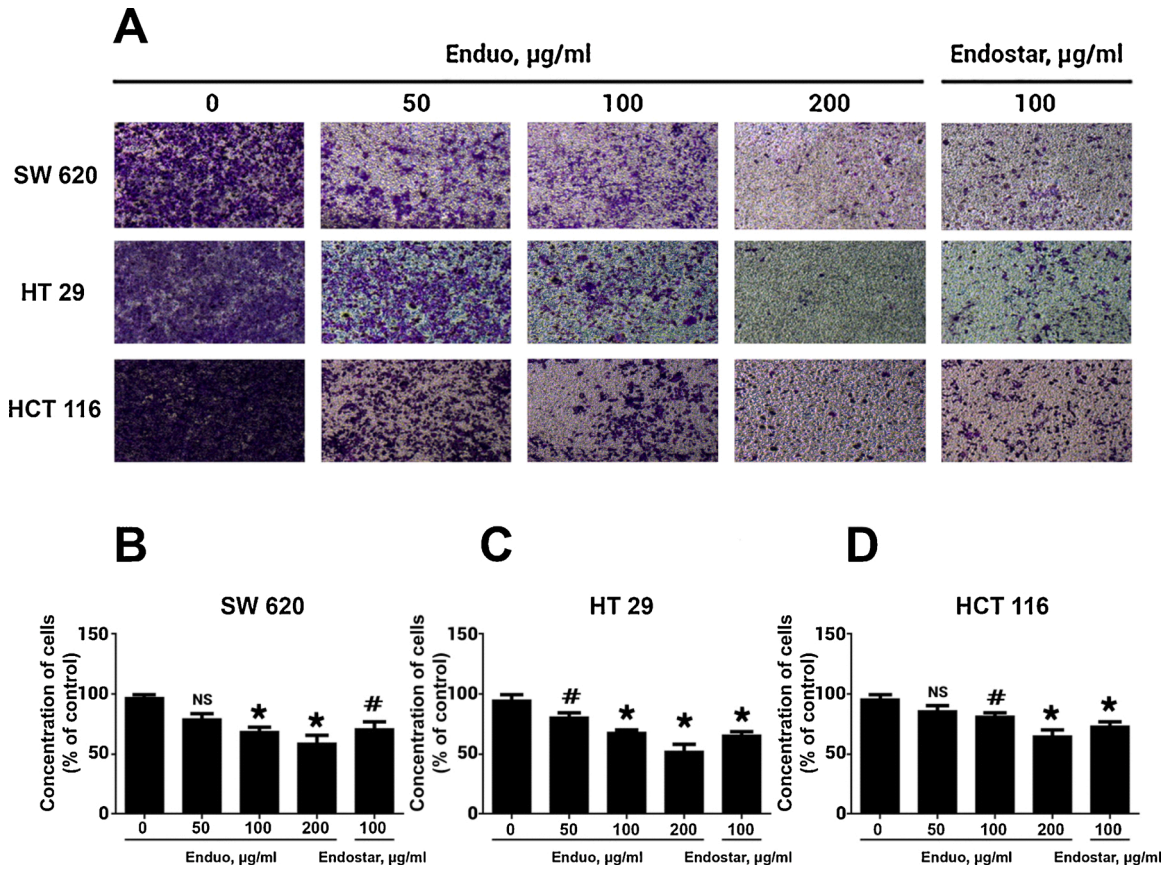

Fig. 3. Inhibitory effect of EnDuo and Endostar on colon cancer migration assessed using the transwell technique (A) (Scale bar 200 mm). The effect of EnDuo and Endostar (0-200 µg/mL) on the migration of SW 620, HT 29 and HCT 116 colon cancer cells. Representation of images captured at three different fields and the results analyzed by Image J software. Proportion of migrated cells of (B) SW 620, (C) HT 29, and (D) HCT 116 cells relative to the control group. The data are presented as the mean ± SD of three independent experiments in triplicate. #P < 0.05 vs. the control, *P < 0.01 vs. the control.

2.5. Transwell migration assay

The transwell migration assay technique was employed to assess the effect of EnDuo on the colon cancer cell lines. A polycarbonate transwell chamber with 8 µm pore diameter (Coming Costar, Cambridge, MA, USA) was used. Colon cancer cells treated with EnDuo (0−200 µg/mL) or Endostar (100 µg/mL) for 24 h were trypsinized and suspended at 1×10^6 cells/mL in RPMI1640 medium without serum as a final concentration. Cell suspension was loaded into the upper chamber, and the medium with 10 % FBS as a chemoattractant was applied to the lower chamber. The setup was incubated at 37 °C for 24 h. After incubation, the non-migrated cells in the upper chamber were removed with a cotton swab. The migrated cells were fixed with 100 % methanol and then stained with 1% crystal violet in 2% ethanol. Images of the stained cells were captured at nine different fields, and the number of cells enumerated and presented as the average cell count. The experiment was repeated at least three different time in triplicated and the results shown as mean ± SD.

2.6. Western blot analysis

The colon cancer cells were seeded into a 6-well plate at a density of 2×10^5 cells/well for 24 h. After acclimatizing, the cells were incubated with different concentrations of EnDuo (0−200 µg/mL) and Endostar (100 µg/mL) for another 24 h. This was followed by collection and lysing of the cells with a cocktail of RIPA cell lysis buffer and a protease inhibitor PMSF. The lysates were incubated for 30 min on ice and centrifuged at 8,000 × *g* for 15 min at 4 °C. The supernatant of the lysates was collected and stored at −80 °C for use in subsequent experiments. Concentration of the protein was determined using the BCA protein assay kit. About 20 µg of the protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted to a polyvinylidenefluoride (PVDF) membrane, blocked with 5% skimmed milk and trice washed with Tris Buffered Saline with Tween 20 (TBST). The membrane was incubated with primary mAb at 4 °C overnight. After washing with TBST, the membrane was incubated in Horseradish peroxidase-COnjugated goat anti-rabbit IgG secondary antibody for 1 h at room temperature. The protein bands were visualized after applying an enhanced chemiluminescence (Odyssey, LI-COR Biosciences, Lincoln, NE, USA).

2.7. Statistical analysis

All data were presented as the mean ± SD of three autonomous experiments performed in triplicate. Statistical analysis was performed by one-way analysis of variance (ANOVA). In all instances, P < 0.05 was considered statistically significant.

3. Results

3.1. EnDuo did not produce significant toxic effects on SW 620, HT 29 and HCT 116 colon cancer cells

To evaluate the effect of EnDuo on cell viability, the CCK-8 assay technique was used. As shown in Fig. 1, EnDuo did not induce significant cytotoxic effect on the colon cancer cells at concentrations 50 µg/mL and 100 µg/mL compared to the control. However, there was a significant inhibitory effect at concentration 200 µg/mL compared to the control.

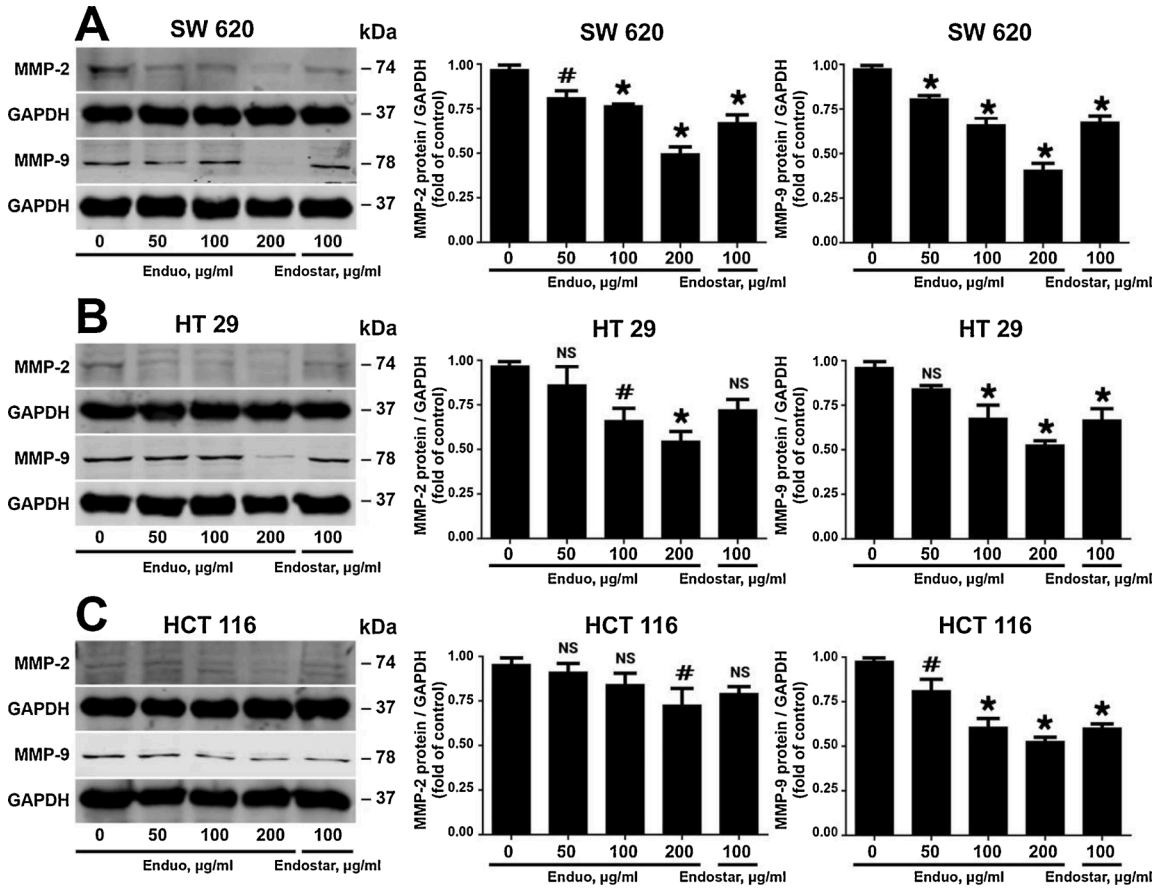

Fig. 4. Effect of EnDuo on the expression of MMP-2 and MMP-9 in colon cancer cells. The inhibitory effect of EnDuo and Endostar (0-200 μg/mL) on the expression of MMP-2 and MMP-9 in.(A) SW 620, (B) HT 29 and (C) HCT 116 cells as assessed by western blot analysis. Corresponding histograms represent the relative expression quantities of the target protein to the control, analyzed by Image J software. The data are presented as the mean ± SD of three independent experiments in triplicate. #P < 0.05 vs. control, *P < 0.01 vs. control.

Comparing the 100 μg/mL Endostar to EnDuo, we observed no significant association between the two agents in terms of cell viability. This observation suggests that EnDuo confers growth restriction on the colon cancer cells in a concentration-dependent manner.

3.2. EnDuo inhibits the migration of colon cancer cells in vitro

Assessment of the relocation and motility of the colon cancer cells in the presence of EnDuo was first demonstrated by the scratch wound assay. As shown in Fig. 2A and B, EnDuo effectively inhibited the migration of SW 620 cells after 48 h of treatment, and exhibited an enhanced migration inhibition after 72 h of treatment. In the case of HT 29 cells, the inhibition of migration by EnDuo was observed as early as 12 h post treatment and was sustained even after 72 h. The migration rate decreased gradually with increasing time and EnDuo concentration in contrast to the control group (Fig. 2C and D). In a similar manner, EnDuo arrested the migration of HCT 116 cells 24 h post treatment and the effect was sustained after 72 h, suggesting EnDuo's potency is time and concentration dependent (Fig. 2F and E). Even though there were variations in the time for significant observable wound closure inhibition, the study proved that EnDuo significantly suppresses the migration of colon cancer cells, and the effect is both time and concentration dependent. Furthermore, we investigated the inhibitory effect of EnDuo on the migration of colon cancer cells by transwell assay as presented in Fig. 3. After 24 h treatment, EnDuo markedly decreased the quantity of migrating cancer cells. This observation was in conformity with the wound scratch test, which testified that EnDuo suppresses the movement of colon cancer cells.

3.3. EnDuo reduces the expression of MMP-2/-9 in colon cancer cells

Matrix metalloproteinases (MMPs) have the potential to degrade extracellular matrix (ECM) to promote EMT of cancer cells. From the MMPs family, MMP-2 and MMP-9 are associated with the metastasis of tumors and are exceptionally expressed [36]. We therefore, studies the expression of MMP-2 and MMP-9 in the current study by western blot to determine the possible anti-invasive activity of EnDuo. As shown in Fig. 4, EnDuo (50−200 μg/mL) decreased the expression of MMP-2 and MMP-9 in SW 620, HT 29 and HCT 116 colon cancer cells concentration-wise.

3.4. EnDuo decreases the phosphorylation of ERK and AKT in colon cancer cells

Studies have shown that the downstream signaling molecules Ras/MEK/ERK and PI3K/AKT pathways are set off by the complex FAK-Src, are is connected to the metastasis and survival of cancer cells [37]. Therefore, the phosphorylation of key molecules, AKT and ERK, in these pathways were determined by western blot. As demonstrated in Fig. 5, the phosphorylation of AKT and ERK was decreased by EnDuo (50−200 μg/mL) concentration-wise. It is important to emphasize that there were no observable contrasts in the total AKT and ERK bands. Generally, the study illustrated that EnDuo may suppress the metastasis of colon cancer cells by hindering the PI3K/AKT and ERK/MAPK pathways.

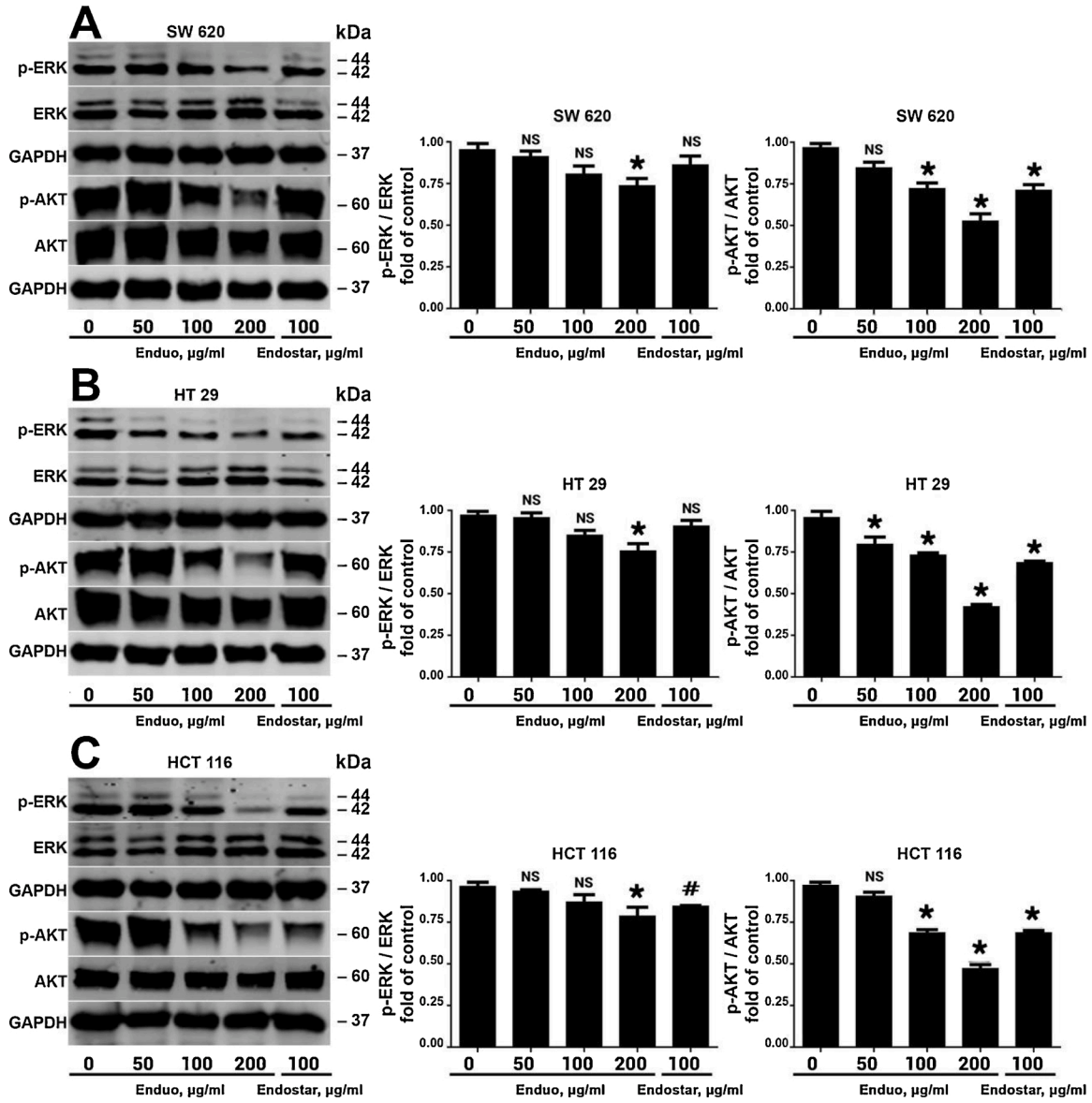

Fig. 5. Effect of EnDuo on the expression and phosphorylation of AKT and ERK in colon cancer cells. The inhibitory effects on the expression levels of phosphorylated and total AKT and ERK in (A) SW 620, (B) HT 29 and (C) HCT 116 colon cancer cells analyzed by western blotting. Corresponding histograms represent the relative expression quantities of the target proteins to the control analyzed by Image J software. The data are presented as the mean ± SD of three independent experiments in triplicate. #P < 0.05 vs. the control, *P < 0.01 vs. the control.

4. Discussion

Metastasis involves diverse, complex and exaggerated mechanisms which result in an uncontrollable cancer cell motility, proliferation, cell adhesion to ECM and ECM proteolysis. Since deaths from colon cancer is significantly ascribed to metastasis, there is a pressing concern globally to discover or create novel therapeutically helpful agents to suppress the metastasis of colon cancer. EnDuo is a biologically active peptide which promises to be a potent antitumor and chemo-preventive agent against malignancies. Nevertheless, information on the inhibitory impacts of EnDuo on the metastasis of malignant colon cells and its mechanism are limited. We have demonstrated in this study that EnDuo stifles the movement of malignant colon cells. In addition, we showed that EnDuo suppresses the expression of MMP-2/-9, and mechanistically, down-regulates the phosphorylation of ERK and AKT. This observation suggests that EnDuo restrains the migration of colon cancer cells by suppressing the PI3K/AKT and ERK/MAPK pathway.

Cell apoptosis is usually induced and controlled by polygenic pathways, such as blockage of cell cycle and expression changes in correlated apoptosis genes. Signaling pathways such as ERK/MAPK and PI3K/AKT play crucial roles in tumor cell development, apoptosis, invasion, progression and metastasis [38]. The PI3K/AKT signaling pathway has been well documented for its major role in breast cancer, lung cancer, cervical cancer, colon cancer and other types of cancers [39–41], and this pathway represents an attractive target for anticancer therapeutics. Activation of AKT is linked to malignancies, demonstrating that p-AKT is an autonomous prognostic marker for cancer patients [42]. MAPK family and its subfamily ERK, can be actuated by various growth factors and cytokines to influence proliferation and apoptosis of cells [43]. Turning on ERK induces the activation many downstream genes including Cyclin D1, which plays a role in the transformation of malignant cell. Most notably, programmed death of cell and growth inhibition of tumor cells ensue following blockage of AKT or ERK signaling pathway [44]. Our findings support these reports as EnDuo inhibited the expression of p-AKT, and p-ERK.

The PI3K/AKT/mTOR signaling hub is fundamental to the

development and support of colorectal cancer cells, and is crucial in the proliferation, protection from apoptosis, angiogenesis and metastasis [45]. The advancement of efficient treatment of colon cancer may require the inhibition of the AKT pathway. Nonetheless, many studies have reported on the potential anticancer agents inhibiting the AKT pathway, and more are yet to successfully complete clinical trials [46–49]. Decreased activation of AKT in growth-retarded tumor cell have been reported by past investigations [46–49]. Additionally, previous investigations have demonstrated that targeting the PI3K/AKT signaling pathway with anti-sense small interfering (si)RNA or small molecule inhibitors results in the suppression of tumorigenesis and tumor invasion [50,51].

The migratory capacity of cancer cells gives them the opportunity to move to surrounding tissues, eventually leading to metastasis. As observed in this study, EnDuo suppressed the ECM proteolytic degradation. MMPs, a family of zinc-containing endopeptidases, play a crucial role in the process of ECM degradation [52]. In this work, it was found that the expression of MMP-2 and MMP-9 in SW 620, HT 29 and HCT 116 colon cancer cells were markedly inhibited by EnDuo. Previous studies have demonstrated that decreased expression of MMP-2 and MMP-9 in cancer cells are strongly identified with the inhibition of PI3K/AKT and ERK, which are FAK downstream targets [53,54]. Enacted AKT prompts the invasion and metastasis of cancer cells by promoting the secretion of MMPs [55,56]. It has been proposed that MAP family kinases are possibly involved in the signaling processes that regulate MMPs, including MMP-9 [57].

In conclusion, this study has shown that EnDuo is capable of inhibiting the migration of cancer cells *in vitro*. Also, EnDuo hinders the expression of MMP-2 and MMP-9, and arrests AKT and ERK phosphorylation. Perhaps, EnDuo exhibits its anti-metastasis property via suppressing the PI3K/AKT/ERK signaling pathway. However, an expanded *in vivo* study will be required to further elucidate the full potential of EnDuo against colon cancer, and possibly other cancers.

Author contributions

EI and BL conceived and designed the study. EI performed the experiments. EI wrote the manuscript in consultation with WW, MA, VP and BL. VP and BL supervised the study. All authors read and approved the manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.

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