Therapeutic role of 2-stearoxyphenethyl phosphocholine targeting microtubule dynamics and Wnt/β-catenin/EMT signaling in human colorectal cancer cells

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Abstract

The inhibition of cell death, perturbation of microtubule dynamics, and acceleration of Wnt/β-catenin/epithelial-mesenchymal transition (EMT) signaling are fundamental processes in the progression and metastasis of colorectal cancer (CRC). To explore the role of 2-stearoxyphenethyl phosphocholine (stPEPC), an alkylphospholipid-based compound, in CRC, we conducted an MTT assay, cell cycle analysis, western blot analysis, immunoprecipitation, immunofluorescence staining, Annexin V/propidium iodide double staining, small interfering RNA gene silencing, a wound-healing assay, an invasion assay, and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay in the human CRC cell lines HT29 and HCT116. stPEPC showed anti-proliferative properties and mitotic cell accumulation via upregulated phosphorylation of BUBR1 and an association between mitotic arrest deficiency 2 (MAD2) and cell division cycle protein 20 homolog (CDC20). These results suggest that activation of the mitotic checkpoint complex and tubulin polymerization occurred, resulting in mitotic catastrophe in HT29 and HCT116 cells. In addition, stPEPC attenuated cell migration and invasion by regulating proteins mediated by EMT, such as E-cadherin and occludin. stPEPC downregulated the protein expression of Wnt3a and phosphorylation of low-density lipoprotein receptor-related protein 6 (LRP6), glycogen synthase kinase 3β (GSK3β), and β-catenin as well as their target genes, including cMyc and cyclin D1, in CRC cells. stPEPC may be useful for developing new drugs to treat human CRC.

1. Introduction

Colorectal cancer (CRC) is the third most common cause of cancer-related death in both men and women worldwide (Siegel et al., 2020). Although most treatments for CRC, such as surgery, radiotherapy, and chemotherapy, are effective in the early stages of the disease, they are futile for metastatic CRC, leading to a steep decline in the 5-year survival rate for CRC from ~64–12% (Siegel et al., 2020). CRC-targeted therapy is a selective approach that can extend the overall survival of patients with CRC. Anti-angiogenic drugs, epidermal growth factor receptor blockers, and multiple kinase inhibitors have resulted in small but consistently improved clinical outcomes. However, current targeted therapies frequently cause adverse gastrointestinal and hematological effects (Raskov et al., 2014; Recondo et al., 2014) and offer limited clinical benefits (Curtin, 2013; Raskov et al., 2014; Sridharan et al., 2014). Accordingly, safer, more effective, and affordable therapeutics against metastatic CRC are needed, requiring further investigation to identify agents and approaches for clinical trials.

Wnt signaling is the most common pathway involved in regulating tissue development and maintaining homeostasis, and its dysregulation promotes CRC development (Zhan et al., 2017). Indeed, because Wnt is hyperactivated in approximately 90% of CRC cells (Bordonaro, 2020; MacDonald et al., 2009), inhibition of the Wnt signaling pathway is one strategy for CRC treatment. Classically, Wnt signaling is divided into β-catenin-independent (non-canonical) and dependent (canonical) signaling (Patel et al., 2019). In the absence of Wnt ligands, β-catenin is constantly phosphorylated by a β-catenin destruction complex [comprised of adenomatous polyposis coli protein, axin, casein kinase 1α, and glycogen synthase kinase 3β (GSK3β)], leading to β-catenin ubiquitination, proteasomal degradation, which turn off Wnt-regulated
transcription (MacDonald et al., 2009). In contrast, in the presence of Wnt ligands, Wnt proteins bind to
the seven-pass transmembrane Frizzled receptor and its co-receptor, low-density lipoprotein receptor-
related protein (LRP), resulting in inhibition of the β-catenin destruction complex and promotion of β-
catenin translocation into the nucleus. These steps lead to the activation of Wnt targetted gene
expression, including cyclin D1 and cMyc (He and Tang, 2020; Song et al., 2009). Components of the Wnt
signaling pathway play crucial roles in the mitosis of CRC cells by regulating microtubule dynamics,
spindle formation, and centrosome division (Wu et al., 2017). Thus, Wnt/β-catenin-targeted therapies are
promising candidates against CRC progression involving tumor growth, invasion, and metastasis.

Compounds structurally related to alkylphospholipids (APLs) are a new class of drugs that do not directly
interact with DNA but act on cell membranes and accumulate to interfere with lipid metabolism and
signaling pathways (Patel et al., 2002). In addition, phosphorylation of Akt can be inhibited by introducing
cyclopentane or pyrrolidine into the structure of APL, suggesting its potential as an anticancer agent
(Alam et al., 2012; Alam et al., 2013). Edelfosine (first-generation APL) and ilmofosine (second-generation
APL) possess potent antitumor activities but have limited clinical applications (Vink et al., 2007). In
addition, although APL hexadecylphosphocholine (miltefosine) is clinically effective in patients with
metastatic breast cancer and cutaneous lymphomas (van der Luit et al., 2007), the anti-proliferative
effects and molecular mechanisms of structurally related APL derivatives remain unclear. Herein, the
antitumor activity and underlying molecular mechanisms of the anti-proliferative properties of the
synthetic APL derivative 2-stearoxyphenethyl phosphocholine (stPEPC) were investigated in CRC cells.

2. Methods and materials

2.1. Preparation of 2-stearoxyphenethyl phosphocholine (stPEPC)

2-Stearoxyphenethyl phosphocholine (stPEPC, Fig. 1) was prepared as reported previously (Hassan et al.,
2023). Briefly, 2-stearoxyphenethyl alcohol was underwent domino reaction with 2-chloro-2-oxo-1,3,2-
dioxaphospholane then trimethylamine to afford stPEPC. Spectroscopic data was identical to previously
reported data (supplementary method).

2.2. Cell culture

Human CRC cell lines HT29 and HCT116 were obtained from the Korea Cell Line Bank (KCLB, Seoul,
Korea). All cells were cultured according to the cell culture information of KCLB guideline.

2.3. MTT assay

Cytotoxicity was evaluated using an MTT assay. Briefly, 100 µL of cell suspension (3 × 10^5/mL) was
incubated in a 96-well plate for 24 h, followed by treatment with 100 µL of stPEPC (0, 6.25, 12.5, or 25
µM). After 48 h, 20 µL of MTT stock solution (5 mg/mL) was added and incubated at 37°C for further 4 h.
The formed formazan blue was dissolved in 200 µL of dimethyl sulfoxide (DMSO) and the optical density was measured at 540 nm.

2.4. Analysis of cell cycle progression

Cells were seeded in 60 mm plates at a density of 3 x 10^5/mL and incubated with stPEPC (6.25, 12.5, or 25 µM) for 24 h. Next, cells were washed with phosphate-buffered saline (PBS) and fixed in cold 70% ethyl alcohol for 24 h at 4°C. Then, cells washed with PBS and were incubated with RNase A for 30 min and stained with 400 µL propidium iodide (PI, 10 mg/mL) for 30 min at 25°C. The stained cells were analyzed by flow cytometer (Cytomics FC 500, Beckman Coulter, CA, USA).

2.5. Western blot analysis

To determine changes in protein expression, cells were extracted by PRO-PREPTM protein lysis buffer (Intron Biotechnology, Seongnam, Korea) for 30 min 4°C. Protein concentration was quantified by the Bradford assay. The proteins (30 µg) were separated by SDS-PAGE gel electrophoresis. Separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes and incubated with primary antibody [1:1,000 diluted by 2.5% skim milk in 0.1% Tween 20/Tris-buffered saline (T/TBS)] for 24 h at 4°C. The immunoblotted membrane was washed with T/TBS solution and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2,000 diluted by 2.5% skim milk in T/TBS) for 2 h at 25°C. They were again washed with T/TBS solution and detected using an enhanced chemiluminescence detection system (Amersham, Buckinghamshire, UK).

2.6. Immunoprecipitation

Cells were lysed in RIPA buffer containing 1mM phenylmethylsulfonyl fluoride (PMSF) and 1× protein inhibitor cocktail by sonication. Antibodies were placed in the PureProteome™ protein G magnetic beads (Millipore crop, Billerica MA, USA) from which the storage buffer was removed and slowly rotated for 4 h. The bead combined with antibodies was washed and incubated in rotation with quantified proteins for 24 h. Immunoprecipitated proteins were separated by 8% SDS-PAGE gel electrophoresis and then analyzed by western blotting.

2.7. Immunofluorescence staining

Cells were washed with PBS and fixed in situ with 90% methanol at 37°C for 1 h. Cells were again washed with PBS and incubated in a blocking solution (10% normal goat serum in 0.3% Triton-X/PBS) for 1 h. Then cells were reacted with anti-α-tubulin antibody (diluted 1:100, Santa Cruz, CA, USA) for 24 h at 4°C. The primary antibody was removed by washing solution (0.3% Triton-X/PBS) and incubated for 1 h with Alexa 594-conjugated anti-mouse secondary antibody (diluted 1:100). Fluorescent signals were imaged using a confocal laser scanning microscope (NANOSCOPE systems, Seoul, Korea).

2.8. Preparation of monomeric and polymeric tubulin

Cells were suspended with monomer extraction buffer [20 mM piperazine-N,N-bis(2-ethanesulfonic acid) (pH 6.8), 0.14 M NaCl, 1 mM MgCl₂, 1 mM ethylene glycol-bis(2-aminoethylether)-N,N,N,N-tetraacetic
acid, 0.5% NP-40, 0.5 mM PMSF] and then centrifuged at 15,000 × g for 30 min at 25°C. The supernatant containing monomeric tubulin was transferred to a new tube and the remaining polymeric tubulin material was dissolved in PRO-PREPTM protein lysis buffer (Intron Biotechnology, Seongnam, Korea) at 4°C for 30 min. Monoer-c-and polymeric tubulin fractions were quantified by the Bradford assay and tubulin expression level was determined by western blot analysis.

2.9. Annexin V and PI double staining

Cells were reacted in 1 mL of binding buffer and then stained with FITC-conjugated Annexin V and PI solutions in the dark for 15 min. The stained cells were analyzed by flow cytometer (Cytomics FC 500, Beckman Coulter, CA, USA).

2.10. Wound Healing Assay

After the preparation of the confluent cell monolayer, a cell-free gap was made by mechanical scratching with a micropipette tip in the center of the culture vessel. Then cells were washed with medium and treated with stPEPC (25 µM) for 24 h. Cell migration was assessed by measuring the scratch area.

2.11. Invasion Assay

In the transwell (Corning, NY, USA), the insert chamber was pre-coated with matrigel (Thermo Fisher Scientific, MA, USA) and inoculated with suspended cells. Downwells were treated with stPEPC (25 µM) and incubated for 48 h. Cells were then fixed and stained using Diff Quik kit (Sysmex, Kobe, Japan) from Sysmex Corporation. The cells on the upper part of the insert chamber were removed with a cotton swab, and the cells under the insert chamber were observed with a microscope (Olympus, Tokyo, Japan).

2.12. Transfection

Mitotic arrest deficiency 2 (MAD2) siRNA (Santa Cruz, CA, USA) was transfected into the cells using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) and then the cells were treated with stPEPC for 18 h. After siRNA transfection, cellular expression of MAD2 was determined by western blot analysis.

2.13. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

Cell transfected with siRNA were fixed with 4-formaldehyde for 1 h. The cells were washed with PBS and treated with methanol for 10 min. Then, the cells went under the permeabilization process on ice for 2 minutes and the TUNEL mixture was reacted according to the manufacturer’s instructions (In situ cell death detection kit, POD, Roche, Germany). The stained cells were detected by an Olympus IX51 fluorescence microscope (Olympus, Tokyo, Japan).

2.14. Statistical Analysis

Data are represented as the mean ± SD of triplicate experiments. Statistical significances were identified using analysis of variance (ANOVA) and Dunnett’s post hoc test, and \( p \)-values of less than 0.05 were considered as statistically significant.
3. Results

3.1. stPEPC induces G2/M arrest and suppresses phosphorylation of Akt in human CRC cells

To assess the cytotoxic effect of stPEPC, lung (A549 and H358 cells), colon (HCT116 and HT29 cells), and cervix (HeLa and SiHa cells) cancer cells were evaluated in an MTT assay following treatment with variable concentrations of stPEPC (3.125–100 µM) for 48 h to determine the half-maximal inhibitor concentration (IC50). Among these cancer cells, colon cancer cells showed the highest sensitivity to stPEPC, whereas normal cells (MRC5 and IOSE-80PC cells) showed higher IC50 values (68.91 and 72.03 µM, respectively [Table 1]).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>IC50 (µM) 1</th>
<th>Ah-yn-1</th>
<th>Miltefosine</th>
</tr>
</thead>
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<tr>
<td>A549</td>
<td>Human lung adenocarcinoma</td>
<td>55.60 ± 5.76</td>
<td>&gt; 100</td>
<td></td>
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<tr>
<td>H358</td>
<td>Human lung carcinoma</td>
<td>48.81 ± 5.88</td>
<td>&gt; 100</td>
<td></td>
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<tr>
<td>HCT116</td>
<td>Human colorectal carcinoma</td>
<td>14.04 ± 4.41</td>
<td>76.96 ± 6.53</td>
<td></td>
</tr>
<tr>
<td>HT29</td>
<td>Human colorectal adenocarcinoma</td>
<td>8.07 ± 5.69</td>
<td>70.38 ± 7.18</td>
<td></td>
</tr>
<tr>
<td>HeLa</td>
<td>Human cervical carcinoma</td>
<td>61.99 ± 5.76</td>
<td>71.29 ± 5.88</td>
<td></td>
</tr>
<tr>
<td>SiHa</td>
<td>Human cervical carcinoma</td>
<td>52.63 ± 4.92</td>
<td>&gt; 100</td>
<td></td>
</tr>
<tr>
<td>MRC5</td>
<td>Human fetal lung fibroblast</td>
<td>68.91 ± 7.63</td>
<td>59.47 ± 4.35</td>
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</tr>
<tr>
<td>IOSE-80PC</td>
<td>Human normal ovarian surface epithelial</td>
<td>72.03 ± 8.91</td>
<td>31.86 ± 3.46</td>
<td></td>
</tr>
</tbody>
</table>

1 IC50 is defined as the concentration that results in a 50% decrease in the number of cells compared with that of the control cultures in the absence of stPEPC. The values represent the mean of three independent experiments.

As shown in Fig. 2A, stPEPC reduced cell viability in time- and concentration-dependent manners after 48 h, with IC50 values of 8.07 ± 5.69 and 14.04 ± 4.41 µM against HT29 and HCT116 cells, respectively. To determine how stPEPC inhibited cell cycle progression, the DNA content was analyzed using PI staining with flow cytometry. stPEPC treatment resulted in a marked accumulation of G2/M phase cells and a decrease in G0/G1 phase cells, suggesting that stPEPC induces G2/M phase arrest in both HT29 and HCT116 cells (Fig. 2B). In addition, stPEPC, an alkyl phospholipid, strongly inhibited Akt phosphorylation in both CRC cell lines (Figure S1).
3.2. stPEPC induces mitotic cell accumulation in human CRC cells

As the G₂/M transition modulates the cyclin B1/cdc2 complex protein through p-cdc25c (Ser216) (Chiang et al., 2021; Wu et al., 2017), we analyzed how stPEPC affects G₂/M transition-related regulatory proteins using western blot analysis. As shown in Fig. 3A, stPEPC induced phosphorylation of cdc2 (Thr161), dephosphorylation of cdc2 (Tyr15) and cdc25c (Ser216), and upregulation of cyclin B1 protein expression, indicating activation of the cyclin B1/cdc2 complex. Furthermore, phase-contrast microscopy showed that stPEPC-treated cells were more rounder than control cells (Fig. 3B). Moreover, stPEPC induced phosphorylation of histone H3 (Ser10), a well-established marker of mitosis, in a concentration-dependent manner (Fig. 3C). These data reveal that stPEPC caused cells to enter the mitotic phase and led to subsequent mitotic cell accumulation, representing mitotic arrest in HT29 and HCT116 cells.

3.3. stPEPC induces the activation of spindle assembly checkpoint (SAC) and the complex formation of MAD2 with CDC20 in human CRC cells

Spinal assembly checkpoint (SAC) has been implicated in the regulation of chromosomes that are unattached to the mitotic spindle, leading to mitotic inhibition to prevent errors in chromosome segregation (Lara-Gonzalez et al., 2021). In addition, SAC regulation is affected by the phosphorylation of aurora kinase, which contributes to mitotic checkpoint responses (Goldenson and Crispino, 2015). Therefore, we examined whether stPEPC-induced mitotic arrest is regulated by SAC components in CRC cells. As shown in Fig. 4A, stPEPC upregulated the phosphorylation of BUBR1 and aurora kinases A/B/C and the expression level of MAD2, whereas the expression level of CDC20 was decreased. In addition, stPEPC induced mitotic checkpoint complex binding between CDC20 and MAD2, indicating inhibition of anaphase-promoting complex/cyclosome (APC/C)-dependent cyclin B1 degradation (Fig. 4B). To confirm whether stPEPC-induced mitotic arrest was associated with SAC, we evaluated the phosphorylation of histone H3 on stPEPC in HT29 and HCT116 human CRC cells after MAD2-specific siRNA transfection. Cells transfected with MAD2-specific siRNA showed reduced expression levels of MAD2 in western blot analysis (Figure S2). As shown in Fig. 4C, treatment with stPEPC did not lead to phosphorylation of histone H3 in MAD2-suppressed cells through siRNA interference compared to the effects in stPEPC-treated control cells. These results indicate that stPEPC-induced accumulation of mitotic cells depends on SAC activation, which leads to perturbation of anaphase progression in HT29 and HCT116 cells.

3.4. stPEPC induces microtubule stabilization in human CRC cells

The mitotic spindle consists of microtubules, and microtubule dynamics involving movement and stabilization are involved in all mitotic processes (Goodson and Jonasson, 2018). To investigate whether stPEPC affects microtubule dynamics leading to mitotic arrest, immunocytochemical analysis was performed. As shown in Fig. 5A, stPEPC increased the fluorescence intensity of α-tubulin, similarly to the effects of paclitaxel (a microtubule polymerizing agent), whereas nocodazole (a microtubule
A depolymerizing agent suppressed the fluorescence intensity of α-tubulin in CRC cells. These results show that stPEPC promotes microtubule polymerization and maintains its stability in CRC cells. To confirm stPEPC-induced microtubule polymerization, western blot analysis was performed using the monomer and polymer fractions from stPEPC-treated cells. Compared to the effects of paclitaxel, stPEPC elevated the expression of polymer-tubulin and reduced the expression of monomer-tubulin in a concentration-dependent manner in both CRC cell lines (Fig. 5B). These results indicate that stPEPC induces the polymerization and stabilization of microtubules, leading to mitotic arrest in CRC cells.

3.5. stPEPC-induced mitotic catastrophe depends on SAC activation in human CRC cells

Cells arrested in mitosis for a prolonged period by anti-microtubule chemotherapeutic agents can undergo a form of apoptotic cell death known as mitotic catastrophe (Hain et al., 2016). To investigate whether stPEPC induces cell death after mitotic arrest, we examined apoptotic cell death and related protein expression using PI and Annexin V double staining and western blot analysis, respectively, in HT29 and HCT116 cells. As shown in Fig. 6A, stPEPC treatment markedly increased the percentage of annexin V-positive cells in a concentration-dependent manner. In addition, stPEPC treatment upregulated the cleaved forms of caspase 3 and PARP and reduced Bcl2 protein expression (Fig. 6B). To verify that stPEPC-induced mitotic catastrophe resulted from mitotic arrest, we investigated whether stPEPC-induced apoptotic cell death depended on SAC activation. As shown in Figs. 6C and D, transfection with MAD2 siRNA reduced stPEPC-induced cleavage of caspase 3 and poly (ADP-ribose) polymerase, as well as DNA fragmentation, compared to the effects in non-transfected stPEPC-treated control cells. These results suggest that stPEPC-induced mitotic catastrophe was due to mitotic arrest caused by SAC activation in HT29 and HCT116 cells.

3.6. stPEPC attenuates cell migration and invasion by regulating epithelial to mesenchymal transition (EMT)-mediated proteins in human CRC cells

In CRC, epithelial-mesenchymal transition (EMT) drives cellular migration, which is characterized by the loss of cell-cell adhesion, leading to tight junction dissolution, disruption of apical-basal polarity, and reorganization of the cytoskeletal architecture; these effects are associated with invasive properties or a metastatic phenotype (Vu and Datta, 2017). Therefore, we analyzed whether mitotic cell death induced by stPEPC was related to the disturbance of metastatic progression in CRC. Our data revealed that treatment with stPEPC increased the expression levels of epithelial markers in a dose-dependent manner, including E-cadherin and occludin (Fig. 7A), and reduced migration compared to the effects in vehicle-treated CRC cells at 24 h (Fig. 7B). In addition, stPEPC significantly decreased the invasive ability of HT29 and HCT116 cells through Matrigel-coated Transwell polycarbonate filters compared to that of vehicle-treated cells at 48 h (Fig. 7C). Tubulin polymerization was observed at 12 h after stPEPC treatment, mitotic arrest
occurred at 24 h, and mitotic catastrophe was induced at 48 h after SAC activation, suggesting that inhibition of CRC metastasis after attenuating cell migration and invasion through the regulation of EMT protein expression is related to mitotic catastrophe in stPEPC-treated CRC cells.

3.8. stPEPC affects Wnt/β-catenin signaling pathway and Wnt target genes in human CRC cells

As the Wnt/β-catenin/EMT signaling pathways are involved in the development, progression, metastasis, and resistance of CRC (Nie et al., 2020), we analyzed the repressive effects of stPEPC on the Wnt signaling cascade in HT29 and HCT116 cells. As shown in Fig. 8A, treatment with stPEPC downregulated the levels of Wnt3a protein expression and phosphorylation of LRP6, GSK3β, and β-catenin, suggesting that the absence of Wnt ligand promoted β-catenin degradation through the ubiquitination-proteasome system (Tauriello and Maurice, 2010). These results confirmed that Wnt target genes, such as cMyc and cyclin D1, were downregulated because β-catenin was degraded when the Wnt signaling pathway was inhibited, resulting in its failure to function as a transcription factor (Fig. 8B).

4. Discussion

Effective new therapies for CRC are urgently needed (Zhou et al., 2021). Characteristics of cancer include sustained proliferation, evasion from growth suppressors, resistance to cell death, immortalized replication, activated invasion, and metastasis (Hanahan, 2022). Although improved therapeutic strategies are being applied in clinical practice, studies are required discover new and effective drugs for CRC treatment because current drugs can cause adverse effects and also lead to drug resistance (Zhang et al., 2021). Although some APL derivatives have been reported to inhibit the proliferation of prostate and CRC cells (Gao et al., 2011; Leroy et al., 2003; Park et al., 2021), the molecular mechanism by which stPEPC induces cell death remains unclear. In this study, we investigated whether stPEPC can be used as a chemotherapeutic agent for CRC by evaluating mitotic arrest, apoptosis, and Wnt/β-catenin/EMT signaling mechanisms in HT29 and HCT116 cells.

Cell cycle checkpoints are critical surveillance factors that halt the progression of cell division, leading to inhibition of tumor cell proliferation, metastasis, and recurrence (Jakowlew, 2006). Among the cell cycle checkpoints, Cdc2 is a cyclin-dependent kinase 1 responsible for G2 entry into mitosis and is activated by phosphorylation at Thr161 and dephosphorylation at Tyr15. Cdc2 interacts with cyclin B1 to form the cdc2/cyclin B1 complex, which plays an essential role in cell cycle regulation as a checkpoint that triggers cell cycle progression from G2 to M phase (Allan and Clarke, 2007). During cell cycle regulation, cdc25c allows the progression to mitosis when the cdc2/cyclin B1 complex is formed (Yan and Peng, 2015). In the present study, although stPEPC regulated cell cycle progression from G2 to M phase by activating the cdc2/cyclin B1 complex, stPEPC induced the phosphorylation of histone H3 (a mitotic index marker), resulting in mitotic cell accumulation, indicating mitotic arrest. During mitosis, SAC, a cell cycle checkpoint, plays a pivotal role in mediating spindle microtubule attachment and chromosome...
in the motor nucleus chromosomes (Nakano et al., 2012). In the early stages of mitosis, improper spindle assembly generates unattached kinetochores that catalyze the formation of the mitotic checkpoint complex (MCC) composed of mitotic kinases MAD2, BUBR1, BUB3, and CDC20, resulting in inhibition of APC/C activation (Orr-Weaver and Weinberg, 1998). MAD2, the first SAC component, binds to CDC20 to form the MCC precursor, which then binds to BUBR1-BUB3, thereby forming MCC (Lara-Gonzalez et al., 2012). We found that stPEPC induced SAC activation and the association of MAD2 with CDC20, suggesting that the MCC precursor was produced, leading to the inactivation of APC/C and the failure of anaphase entry in stPEPC-treated CRC cells.

Microtubules of the mitotic spindle have important regulatory functions for various adhesive molecules, signaling receptors, and oncoproteins (Cermak et al., 2020). Therefore, these microtubule functions are well-established targets for anti-cancer therapies (Huang et al., 2020). Anti-tubulin agents are broadly classified as either microtubule-destabilizing agents or microtubule-stabilizing agents according to their ability to interfere with the dynamic equilibrium of microtubule polymerization or depolymerization (Barreca et al., 2020). For instance, taxane stabilizes microtubules, whereas the vinca alkaloid derivatives and colchicine destabilize microtubules; both types of microtubule poisons prevent all attachments to kinetochores following activation of the mitotic checkpoint involving SAC (Song et al., 2020). In the present study, stPEPC treatment increased the immunofluorescence intensity of microtubules, similar to PTX treatment (a representative stabilizing agent), indicating that microtubule polymerized during mitosis. Mitotic arrest, caused by persistent activation of SAC and chromosome misalignment, eventually leads to non-canonical programmed cell death, known as mitotic catastrophe. Indeed, antimitotic drugs such as geraniin, docetaxel, and vincristine (Pan et al., 2022), which disrupt mitotic progression through SAC activation and result in mitotic catastrophe, play important roles in the treatment of CRC. Despite the constant development of new-generation antimitotic agents, these drugs exhibit limitations such as low efficacy and drug resistance due to mitotic slippage (Komlodi-Pasztor et al., 2012; Weaver and Cleveland, 2005). As defective cells adapt and survive during mitotic slippage, the development of new antimitotic agents should focus on escaping this process before mitotic catastrophe occurs (Mc Gee, 2015). In this study, stPEPC induced cell death involving externalization of PS, DNA fragmentation, and activation of the caspase family after mitotic arrest, whereas gene silencing of MAD2 significantly disturbed stPEPC-induced cell death in CRC cells. Based on these results, stPEPC induced mitotic catastrophe without mitotic slippage via MCC precursor formation, suggesting that stPEPC can be developed as a new-generation antimitotic agent against CRC.

EMT is constitutively promoted by β-catenin signaling during CRC progression and metastasis. β-Catenin is involved in the formation of adherens junctions by binding to the distal region of E-cadherin; however, it can also induce EMT when released from the functional relationship between E-cadherin and β-catenin (Gumbiner, 2005). During EMT progression, epithelial tumor cells undergo distinct morphological and phenotypic alterations, including loss of tight junctions and cell polarity, leading to perturbation of cell-cell interactions, rendering cells more invasive into surrounding tissues (Kim et al., 2019). Particularly, loss or downregulation of E-cadherin and the ability of cell-cell adhesion molecules to inhibit invasion and metastasis are considered as a fundamental and critical step in EMT (Suman et al., 2014). In the
present study, stPEPC inhibited EMT progression via upregulation of E-cadherin and modulation of tight junction proteins (occludin and claudin 2) to prevent the migration and invasion of CRC cells. In addition, we observed downregulation of Wnt3a and a reduction in LRP6, GSK3β, and β-catenin phosphorylation levels in stPEPC-treated CRC cells. Based on our data, stPEPC disturbs EMT progression by inhibiting Wnt/β-catenin signaling; however, further study is required to clarify the relationship between EMT and Wnt/β-catenin signaling by analyzing the effect of stPEPC on the formation of E-cadherin-β-catenin complex in CRC.

5. Conclusions

stPEPC-induced mitotic catastrophe was caused by microtubule polymerization and SAC activation. It may be possible to prevent metastasis by blocking cell migration and invasion through Wnt/β-catenin/EMT signaling in CRC cells. stPEPC shows potential for the development of new drugs for treating human CRC and further in vivo study is needed to confirm these pharmacological efficacies.

Declarations

Author Contributions
Sang-Eun Park: Conceptualization, methodology, validation, formal analysis, investigation, data curation, writing—original draft preparation, visualization. Kyung-Sook Chung: Conceptualization, methodology, validation, investigation, writing—original draft preparation, writing—review and editing, visualization, supervision, project administration. Soo-Yeon Kim: Methodology, validation, formal analysis. Jeong-Hun Lee: Methodology, validation, formal analysis, investigation, data curation, visualization. Ahmed H.E. Hassan: Conceptualization, methodology, validation, resources, data curation, writing—original draft preparation, writing—review and editing. Yong Sup Lee: Methodology and resources. Jae Yeol Lee: methodology and validation. Kyung-Tae Lee: Conceptualization, writing—review and editing, supervision, project administration. All authors have read and agreed to the published version of the manuscript.

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Data availability
The data and material used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Conflicts of Interest
The authors declare no conflict of interest.

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**Figures**

*Figure 1*

Chemical structure of stPEPC
Figure 2

Effect of stPEPC on cell viability and cell cycle in HT29 and HCT116 cells. (A) Cells were treated with indicated concentrations of stPEPC for 24 and 48 h, and then cell viability was assessed by MTT assay. Data are presented as the mean ± SD of three independent experiments. *p < 0.05, **p < 0.01, and ***p < 0.001 vs control cells. (B) After treatment with stPEPC for 24 h, the cell cycle distribution was determined by PI staining assay using flow cytometry. PI, propidium iodide; stPEPC, 2-stearoxyphenethyl phosphocholine.
Figure 3

Induction of mitotic arrest-related proteins in stPEPC-treated HT29 and HCT116 cells. (A) Cells were treated with indicated concentrations of stPEPC for 6 h, and the expression levels of cell cycle-regulating proteins were determined using western blot analysis. β-Actin was used as an internal control. (B) After exposing the cells to stPEPC (25 μM) for 24 h, morphological changes were detected using phase-contrast microscopy (original magnification ×200). (C) Cells were treated with the indicated concentrations of stPEPC for 6 h and then harvested to prepare total cell lysates. The expression level of p-histone H3 was detected using western blot analysis, with β-actin as an internal control. stPEPC, 2-stearoxyphenethyl phosphocholine.
**Figure 4**

Regulation of SAC activity by stPEPC in HT29 and HCT116 cells. (A) Cells were treated with the indicated concentration of stPEPC for 12 h and then harvested to prepare total cell lysates. The expression levels of cell cycle-regulating proteins were determined using western blot analysis. β-Actin was used as an internal control. (B) Cells were treated with stPEPC for 12 h and then lysed with RIPA buffer. The cell lysates were immunoprecipitated with anti-CDC20, followed by western blot analysis using antibodies against MAD2. (C) Cells were transfected with MAD2 siRNA or non-specific control (CON) siRNA and then treated with or without stPEPC (25 μM) for 24 h. The cells were harvested to prepare total cell lysates, and the protein expression levels were determined by western blot analysis. IB, immunoblot; IP, immunoprecipitation; SAC, spindle assembly checkpoint; stPEPC, 2-stearoxyphenethyl phosphocholine.
Figure 5

Effects of stPEPC on microtubule polymerization in HT29 and HCT116 cells. (A) Cells were treated with stPEPC (25 μM), PTX (300 nM), or NOCO (5 μM) for 12 h. PTX and NOCO were used as positive and negative controls, respectively. Cells were stained with anti-α-tubulin monoclonal antibody, and microtubule changes were analyzed under a confocal microscope. (B) Cells were treated with stPEPC (6.25, 12.5, or 25 μM) or PTX (300 nM) for 24 h, and then monomeric or polymeric tubulin was extracted as described in the Materials and Methods. β-Actin was used as an internal control. NOCO, nocodazole; PTX, paclitaxel; stPEPC, 2-stearoxyphenethyl phosphocholine.
Figure 6

Effect of stPEPC on mitotic catastrophe in HT29 and HCT116 cells. Cells were treated with indicated concentrations (6.25, 12.5, and 25 μM) of stPEPC for 48 h, and (A) Annexin V-FITC and PI staining and (B) western blot analysis were conducted. β-Actin was used as an internal control. After transfection with MAD2 siRNA or non-specific control siRNA, the cells were treated with stPEPC (25 μM) for 24 h and then (C) western blot analysis and (D) TUNEL assays were performed. Fluorescence microscopy was used to
identify DAPI-stained cell nuclei (blue) and apoptotic TUNEL-labeled cells (green). FITC, fluorescein isothiocyanate; PI, propidium iodide; siRNA, small interfering RNA; stPEPC, 2-stearoxyphenethyl phosphocholine.

Figure 7

(A)

Before migration | After migration
---|---
0 | 0 | 25

(B)

stPEPC (µM)

0 | 25 µM

HT29

HCT116

600 µm | 500 µm | 500 µm

(C)

<table>
<thead>
<tr>
<th>HT29</th>
<th>HCT116</th>
</tr>
</thead>
<tbody>
<tr>
<td>stPEPC (µM)</td>
<td>0</td>
</tr>
<tr>
<td>E-Cadherin</td>
<td></td>
</tr>
<tr>
<td>Occludin</td>
<td></td>
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<tr>
<td>Claudin 2</td>
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<td>β- actin</td>
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</table>
stPEPC inhibited metastasis progression in HT29 and HCT116 cells. After treatment with or without stPEPC for 24 h, (A) the levels of EMT-related protein expression and (B) migration were assessed using western blot analysis and a wound healing assay, respectively. β-Actin was used as an internal control. Data are presented as the mean ± SD of three independent experiments. *** p < 0.001 vs before migration control cells and ### p < 0.001 vs after migration control cells. (C) After treatment with stPEPC (25 μM) for 48 h, cell invasion was assessed in a transwell invasion assay. stPEPC, 2-stearoxyphenethyl phosphocholine.

**Figure 8**

Effects of stPEPC on the Wnt/β-catenin signaling pathway in HT29 and HCT116 cells (A) After treatment with the indicated concentration of stPEPC, cells were harvested at 1, 2, and 4 h for western blot analysis of Wnt3a, p-LRP6, p-GSKβ, and β-catenin, respectively. (B) After treatment with stPEPC for 12 or 24 h in HT29 or HCT116, respectively, cMyc and cyclin D1 were analyzed using western blotting. β-Actin was used as an internal control. stPEPC, 2-stearoxyphenethyl phosphocholine.

**Supplementary Files**

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