RESEARCH ARTICLE

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Modulation of POPDC1 Expression by Phenothiazine and Trifluoperazine Suppress Colon Cancer Growth and Migration

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Abstract

Objective: The aim of this study was to investigate the effects of CaM antagonist, PTZ, and TFP on cell proliferation and migration of colon cancer cells and its impact on POPDC protein expression. **Methods:** The 50% inhibitory concentration (IC_{50}) of PTZ and TFP in SW1116, SW480, HCT-15, and COLO205 colon cancer cell lines are measured using MTT. Western blot and immunocytochemistry were used to determine the expression of PCNA, cyclin D1 (CD1), and POPDC proteins. Cell migration was observed using a scratch wound-healing assay. **Results:** Treatment with PTZ and TFP inhibited colon cancer cells growth in a dose-dependent manner. PTZ and TFP significantly inhibited the activation of proliferation markers, PCNA and CD1, and the migration of colon cancer cells. Furthermore, POPDC protein was significantly suppressed in all cell types of colon cancer, particularly in SW480. Finally, the CaM antagonist upregulates the *POPDC1* expression in colon cancer cells. **Conclusion:** These findings suggest that CaM antagonists suppress colon cancer cells proliferation via downregulation of CD1 and PCNA. In addition, POPDC protein could be used as a biomarker in colon cancer, and CaM antagonist could be used to regulate *POPDC1* expression. This study suggests that targeting POPDC1 with CaM inhibition could be a potential therapeutic strategy for colon cancer treatment.

Keywords: Phenothiazine- Trifluoperazine- Colon cancer- Migration- POPDC1

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Introduction

Colon cancer is the second most common cause of cancer death globally, with an estimated over 500,000 mortality rates yearly (Kocarnik et al., 2022; Sung et al., 2021). Many nations, including Malaysia, have seen an increase in the prevalence of colon cancer, which has now risen to the third most common cause of cancer mortality, based on the report of the Malaysian National Cancer Registry (MNCR) (Azizah et al., 2019). Despite the remarkable advances in medicine, current treatments have failed to cure the disease and pose adverse effects. Due to relapse and metastasis, the survival rates are also low, with a poor prognosis (Rashid et al., 2009). This necessitates the pursuit of a novel drug capable of successfully targeting cancer cells.

Calmodulin (CaM) is a principal Ca^{2+} sensor protein involved in the modulation of cell growth, migration, invasion, and metastasis (Berchtold and Villalobo, 2014; Villalobo and Berchtold, 2020). In a cell cycle progression, CaM concentrations grow as the cell cycle progresses, peaking at the G1/S transition and remaining high throughout the duration of the cell cycle. In many tumour cells, the level of CaM appears to be higher than in cells from normal tissues. During cell migration, CaM regulates actomyosin contraction via the Ca^{2+}/CaM signalling pathway. CaM's role in cell proliferation and migration has been established in research involving CaM antagonists, which inhibit cell growth and migration of glioblastoma cells, oral cancer cells, and breast cancer cells (Kang et al., 2017; Wu et al., 2016; Zhang et al., 2018). Phenothiazine (PTZ) and trifluoperazine (TFP) are CaM antagonists exhibiting antitumor effects by inhibitory action on CaM when the antagonists bind to CaM and activate the downstream signalling pathway. However, these antagonists' in vitro anti-tumor effects on colon cancer are not well understood.

The Popeye domain containing 1 (POPDC1) is a novel transmembrane protein belonging to the POPDC family that is thought to be a tumour suppressor. A decade ago,

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POPDC1 was discovered as a cell adhesion molecule that presents a vital role in maintaining epithelial integrity and cell movement (Osler et al., 2005). Since then, studies have revealed that downregulation of POPDC1 promotes tumorigenesis in cancer cells. The reduction of POPDC1 protein levels is highly associated with augmented cell proliferation, migration, invasion, metastasis, and poor cancer patient survival rate (Amunjela and Tucker, 2017; Han et al., 2014; Parang et al., 2018; Williams et al., 2011). However, the precise mechanism of POPDC1 in colon cancer cells is still poorly understood. It is thought that targeting and increasing the suppressed expression of POPDC1 might prevent uncontrollable proliferation and migration, making it a potential novel target for inhibiting cancer progression. To the extent of our knowledge, we believe that this is the first study to reveal the potential of CaM antagonist in targeting POPDC1. Hence, in the present study, we aim to investigate the effects of CaM antagonist, PTZ, and TFP on cell proliferation and migration of colon cancer cells and its impact on POPDC protein expression.

Materials and Methods

Cell culture

The human colon cancer cell line SW1116 (#EP-CL-0307), SW480 (#EP-CL-0223), HCT-15 (#EP-CL-0097), and COLO205 (#EP-CL-0066) were obtained from Elabscience, China and CCD-112CoN (normal colon) was a gift from UKM Medical Molecular Biology Institute . The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Nacalai Tesque, Japan) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin (Nacalai Tesque) at 37°C, 5% CO_2.

MTT assay

MTT assay was performed to measure cell proliferation. A total of 5 x 103 cells were seeded in 96-well culture plates and incubated for 24 h. Next, cells were treated with CaM antagonist, phenothiazine (PTZ), and trifluoperazine (TFP) for 24 h. 5 mg/ml of MTT solution [3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenylthetrazoliumbromide] was added to the culture medium and incubated inside 5% CO_2, 37°C incubator. After 2 h, DMSO was added to solubilize formazan crystals. Lastly, absorbance was determined at 560 nm using a GloMax Explorer Multimode microplate reader (Promega).

Western blot

The cells were seeded at 1 x 10^5 cells/ml in 6- well plates and treated with PTZ and TFP for 24 h. The cell lysates were prepared using RIPA lysis buffer (Nacalai Tesque) and protein concentrations were calculated using BSA assay. A total of 10 µg/µl of cell lysate were separated by 12 % SDS-PAGE followed by transfer to a PVDF membrane. The membranes were blocked with 1% BSA in Tris-buffered saline with Tween (TBS-T) for 1 h and then incubated overnight at 4°C with primary antibodies of β-actin diluted (1:5000; #E-AB-20058) from Elabscience, PCNA (1:5000; #GTX100539), CD1 (1:5000; #GTX108624), from GeneTex, and POPDC1 (1:1000; #PA5-80386) from Thermo Fisher Scientific. The next day, membranes were washed with TBS-T prior to incubation with goat anti-rabbit secondary antibody (1:5000; #31462) (Thermo Fisher Scientific) for 1 h at room temperature. Protein bands were visualized with enhanced chemiluminescence (Nacalai Tesque). The protein band intensity was analyzed using ImageJ software (National Institutes of Health, Mary-land, USA) and normalized with β-actin as loading control.

siRNA Transfection

SW480 cells were grown in a complete medium until 80% confluency was reached. Cells were serum-starved for 30 min before transfection using siRNA targeting POPDC1 (BVES siRNA) and non-targeting siRNA (control siRNA) (Santa Cruz Biotechnology). Transfection was performed using jetPRIME® (Polyplus-transfection S.A, Illkirch, France) according to the manufacturer's protocol. The transfection efficiency was measured via western blot 24 h after transfection. Scratch wound healing assay and CaM antagonists treatment were performed 24 h-post-transfection.

Scratch wound healing assay

The SW480 cells were seeded at 1 x 10^5 cells/ml into 6-well plates and incubated inside a 5% CO_2, 37°C incubator until 80% confluent. Then, cells were subjected to serum starvation over-night before treatment for 24 h. Wounds were made carefully using a 200 µl sterile pipette tip. The suspended cells were washed away, and the medium culture was changed into a low serum media. Wound closure images were photographed at 0 h and 24 h in the same area.

Immunocytochemistry

The cells were seeded at 2 x 10^4 cells/ml on sterile gelatin-coated coverslips and incubated at 5% CO_2, 37°C humidified incubator. Cells were fixed onto coverslips for 10 min using 4% para-formaldehyde and washed with PBS before permeabilization and blocking with 0.05% Triton-X in 1% BSA. Cells were subsequently stained with POPDC1 antibody (1:100) over-night at 4°C. The next day, secondary antibody staining was performed with anti-rabbit IgG-CFL 488 secondary (1:250; #sc516248) and phalloidin CruzFluor™ 594 (1:250; #sc-363795) from Santa Cruz for 1 h at room temperature. Lastly, cells were mounted onto microscope slide with ProLong™ Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific). Fluorescence images were captured with a BX63 automated upright micro-scope (Olympus, Tokyo, Japan) and the fluorescence intensity of POPDC1 was analyzed using ImageJ software. The data was presented as comparative ratio of POPDC1 fluorescence intensity/ overall cell fluorescence intensity.

Statistical analysis

One-way ANOVA with Dunnett's post hoc test or unpaired t-test was performed using GraphPad Prism 8.0 (GraphPad Software, Inc., La Jolla, CA, USA) to evaluate statistical significance. Differences were considered

statistically significant at a P-value <0.05. Statistical significance was classified as follows: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Results

Calmodulin Antagonists Inhibit Colon Cancer Cells Proliferation In Vitro

As shown in Figure 1, cell proliferation of all colon cancer cell lines was inhibited after treatment with PTZ and TFP in a dose-dependent manner. The 50% growth inhibition (IC_{50}) values of PTZ and TFP in SW1116, SW480, HCT-15 and COLO205 are 120 – 175 µM and 39 – 64 µM, respectively. On the other hand, PTZ and TFP showed less inhibition on normal colon viabilities, with IC_{50} > 200 uM. The inhibitory effects of PTZ and TFP on various colon cancer cell lines have previously been evaluated, with different cell lines displaying distinct anti-viability activity (Qian et al., 2019; Xia et al., 2019). PTZ and TFP IC_{50} in cancer cells are consistent with prior investigations, which is less than 200 µM and 100 µM, respectively (Faria et al., 2015; Zhelev et al., 2004). IC_{50} of PTZ and TFP was summarized in Table 1 and used for treatment in the subsequent experiments. The data suggests that TFP more efficiently inhibited cell proliferation than an identical concentration of PTZ.

Table 1. The IC_{50} Values for Each Cell Line. IC_{50} values of PTZ and TFP were presented in micromolar (µM) concentration, and the concentration of CaM antagonist used in subsequent experiments is one-fifth of the IC_{50} value.

Colon cancer cells	IC_{50} (µM)		1/5 IC_{50} (µM)	
	PTZ	TFP	PTZ	TFP
CCD112 (normal colon)	290	220	58	44
SW1116	120	39	24	8
SW480	135	44	27	9
HCT-15	150	53	30	11
COLO205	175	68	35	14

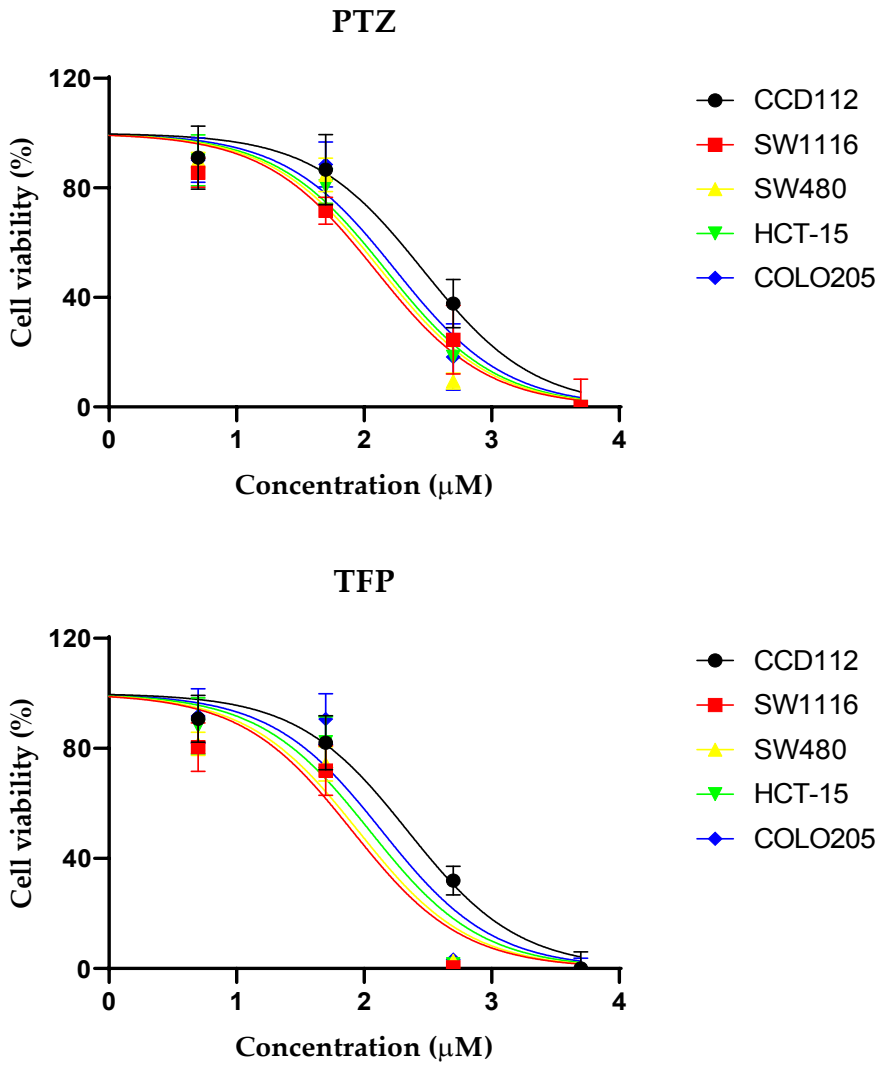

Figure 1. CaM Antagonists Inhibit Colon Cancer Cells Proliferation in Colon Cancer Cell Lines. The effect of CaM antagonists on normal colon CCD112 and different types of colon cancer cells SW1116, SW480, HCT-15, and COLO205 were evaluated by using MTT assay, in which cells were treated with serial dilution of PTZ and TFP for 24 h to obtain the IC_{50} value (half-maximal inhibitory concentration). Data are presented as mean ± SEM (n=3).

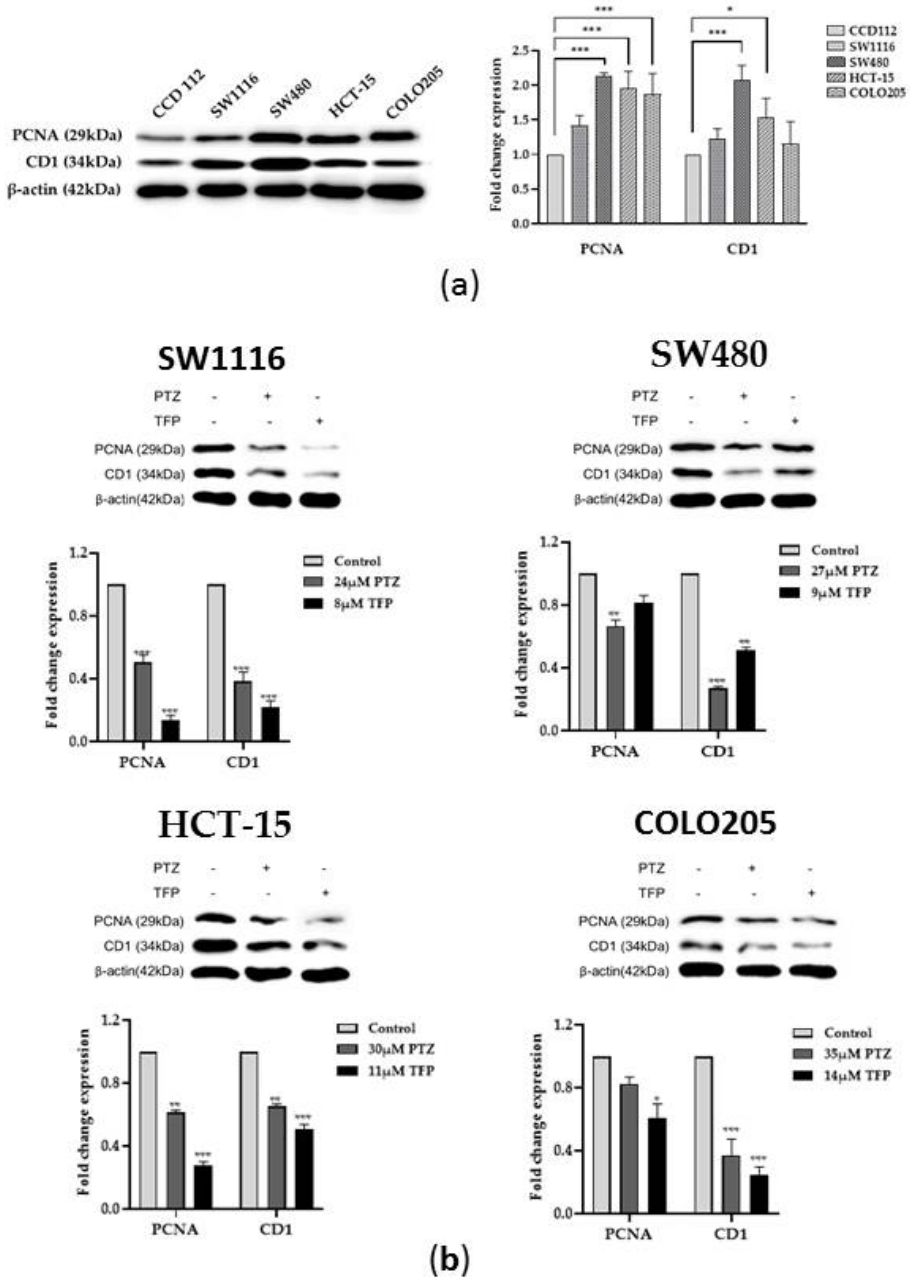

Figure 2. Calmodulin Antagonists Suppress PCNA and CD1 in Colon Cancer Cells. (a) Western blot analysis of PCNA and CD1 expression in CCD112 (normal colon cell) and colon cancer cells (SW1116, SW480, HCT-15, and COLO205) displayed overexpression of colon cancer cells compared to non-tumorigenic cell. (b) The effects of PTZ and TFP on the expression of PCNA and CD1 in PTZ and TFP-treated cells of SW1116, SW480, HCT-15, and COLO205 were determined via western blot and showed suppression in both protein levels. Comparisons between normal cell and cancer cells, and between control and treated groups were conducted using a one-way ANOVA test with Dunnett's post hoc test; *P<0.05, **P<0.005, ***P<0.001. Data are shown as the mean ± SEM (n=3).

CaM Antagonists Suppress the Expression of Proliferation Markers in Colon Cancer Cells

We investigate CaM antagonists' inhibition mechanism on colon cancer cells. PCNA and CD1 are essential cell cycle regulators that is frequently associated with cancer progression, with overexpression detected in many cases (Ding et al., 2020; Mermelshtein et al., 2005; Qie and Diehl, 2016; Ye et al., 2020). We first determine the protein expression of proliferation markers, PCNA and CD1 in SW1116, SW480, HCT-15, and COLO205 cell lines using western blot analysis. As expected, PCNA and CD1 were significantly upregulated in colon cancer cells compared to the normal colon cell (Figure 2a). Next, the effects of PTZ and TFP on the expression of these markers were evaluated. As shown in Figure 2b, PCNA and CD1 expression was reduced in colon cancer cells following treatment were PTZ and TFP. These findings suggest that CaM antagonists might inhibit colon cancer cell proliferation by regulating the cell cycle progression.

POPDC1 Attenuates Colon Cancer Cell Proliferation and Migration

POPDC1 was reported to play an essential role in maintaining epithelial integrity and regulating cell

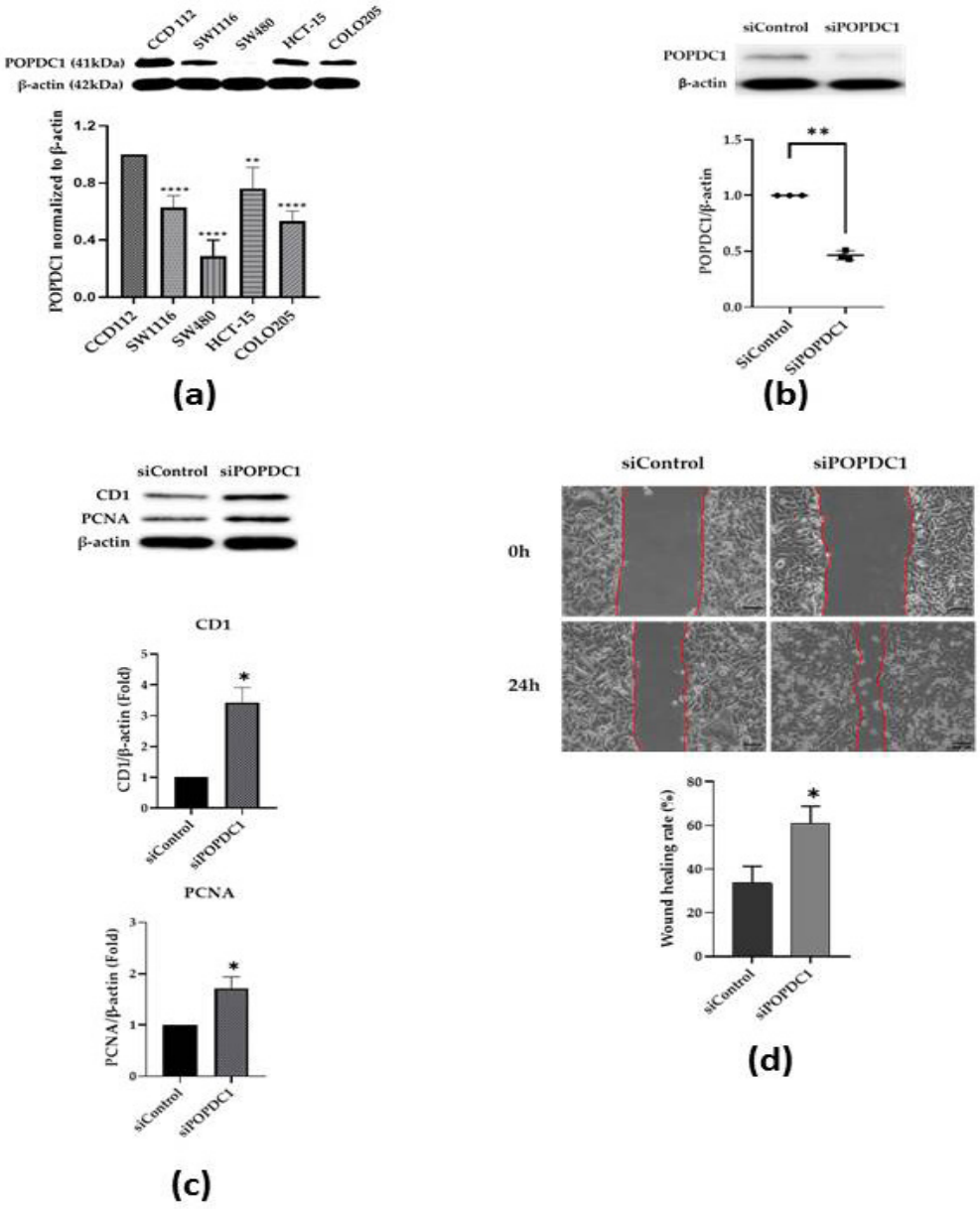

Figure 3. POPDC1 Attenuates Colon Cancer Cell Proliferation and Migration. (a) Western blot analysis of POPDC1 protein expression in CCD112 (normal colon cell) and colon cancer cells (SW1116, SW480, HCT-15, and COLO205) exhibited POPDC1 suppression in colon cancer cells. (b) POPDC1 silencing efficiency in SW480 cells following siPOPDC1 treatment for 24 h were verified with western blot analysis. (c) Western blot analysis of proliferation markers, CD1 and PCNA in response to POPDC1 gene knockdown showed upregulation of PCNA and CD1. (d) Representative cell migration images at time points 0 h and 24 h after wounding and POPDC1 gene knockdown displayed increased rate of migration. Scale bar = 200 µm. Data are shown as the mean ± SEM (n=4). *P<0.05, **P<0.005, ****P<0.0001, significantly different as indicated by one-way ANOVA test with Dunnett's comparison test and unpaired t-test.

movement (Choksi et al., 2018). It was found suppressed in many cancer cells and suggested to be associated with proliferation and migration in cancer (Tucker and Zorn, 2021). First, we performed western blot analysis to confirm the protein expression of POPDC1 in different types of colon cancer (Figure 3a). PODPC1 was markedly expressed in all cell lines with the exception of SW480 cells, where the protein is highly suppressed in the cells (Figure 3a). To further verify the role of POPDC1 on colon cancer cells proliferation and migration, we introduced the short interfering RNA (siRNA) for POPDC1, focusing on SW480 cells. First, we evaluated the silencing efficiency by comparing the POPDC1 protein level following the siRNAs treatment. The gene knockdown of POPDC1 significantly reduced POPDC1 expression as compared to siControl treatment (Figure 3b). Next, we examined the effect of POPDC1 gene knockdown on proliferation markers, CD1 and PCNA. Both CD1 and PCNA proteins were significantly upregulated following POPDC1 silencing, indicating the colon cancer cells regain its proliferative capability in the absence of POPDC1 (Figure 3c). We then confirm the involvement of POPDC1

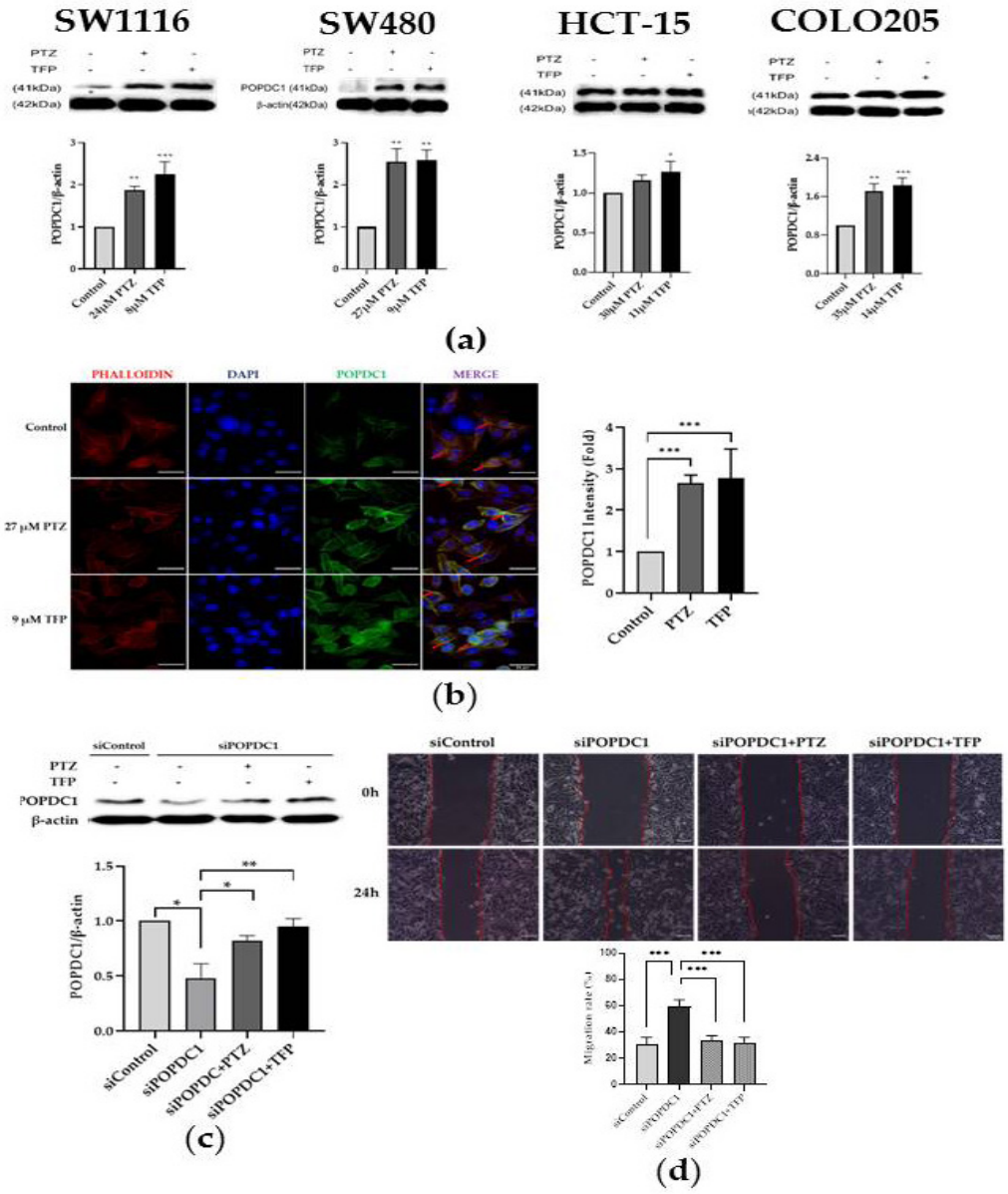

Figure 4. Calmodulin Antagonists Inhibit Colon Cancer Cells Migration by Regulating POPDC1 Protein. (a) Western blot analysis displayed upregulation inPOPDC1 protein expression of colon cancer cell lines (SW1116, SW480, HCT-15, and COLO205) following indicated PTZ and TFP treatment for 24 h. (b) Immunostaining of cell membrane for POPDC1 protein (green) localization, filamentous actin (red) and DAPI (blue) in SW480 cells. Co-localization of PODPC1 and actin are observed and there is increased expression and re-localization of PODPC1 in plasma membrane after treatment with CaM antagonists (red arrow). Scale bar = 20 μm. (c) Western blot analysis of POPDC1 protein following gene knockdown of PODC1 and PTZ (27μM) and TFP (9μM) for 24 h. (d) Representative cell migration images at time points 0 h and 24 h after POPDC1 gene knockdown and CaM antagonist treatment for 24 h. Scale bar = 200 μm. Data are shown as the mean ± SEM (n=4). *P<0.05, **P<0.005, ***P<0.001, significantly different as indicated by one-way ANOVA test with Dunnett's comparison test.

in cancer cell migration using wound healing assay. This study incited the wound by scratching the cells and measuring the migration rate after the siRNA treatments. POPDC1 gene knockdown demonstrated significant increase in cell migration rate as compared to siControl group (Figure 3d). These results suggest the significant involvement of POPDC1 in colon cancer proliferation and migration.

CaM Antagonists Suppress Colon Cancer Cells Migration by Regulating POPDC1 Expression

We then investigate the effect of CaM antagonists on the POPDC1 expression and consequently the migration of colon cancer cells. We first demonstrated that PTZ and TFP induce POPDC1 expression markedly in SW116, SW480, and COLO205 (Figure 4a). This result is further supported by the significant increase in the cell membrane localization of POPDC1 indicated by increased in POPDC1 (green) intensity following PTZ and TFP treatments in SW480 cells as compared to control group (Figure 4b). The localization of POPDC1 (green) is shown lower in control group. As shown in the Figure 3a, protein expression of POPDC1 is highly suppressed in SW480 cells. These results indicated that PTZ and TFP modulate

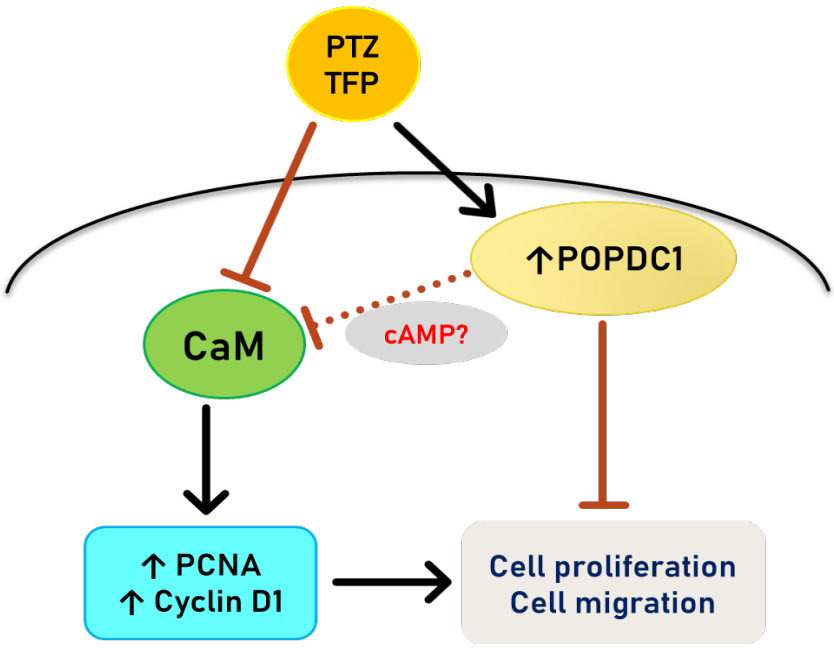

Figure 5. Proposed Mechanism of Calmodulin Antagonist-Mediate Inhibition of Cell Proliferation and Migration in Colon Cancer Cells via POPDC1 Signalling. Calmodulin antagonist blocks CaM, which suppresses PCNA and Cyclin D1 and targets POPDC1, subsequently inhibiting cell proliferation and migration in colon cancer cells.

the activation of POPDC1 in SW480 by changing its localization to the cell membrane. We then verified the effect of PTZ and TFP on POPDC1 activation in SW480. The introduction of siRNA for POPDC1 demonstrated the re-activation of POPDC1 in response to PTZ and TFP treatment (Figure 4c). The role of CaM antagonists on POPDC1 upregulation further confirmed in the wound healing assay. Treatment of PTZ and TFP significantly suppressed PODC1 gene knockdown-induced SW480 cell migration (Figure 4d). These findings indicate that the anti-proliferative activity of CaM antagonists subsequently inhibits the colon cancer cells migration via the modulation of POPDC1 protein.

Discussion

In this present study, we have shown that both CaM antagonists displayed significant anti-proliferative activities in different types of colon cancer cells, with TFP having a greater inhibitory concentration (IC_{50}) than PTZ. The CaM antagonists also displayed less toxicity in non-tumorigenic cells. Moreover, the antagonists suppressed the proliferation markers CD1 and PCNA in colon cancer. CD1 and PCNA are important indicators for tumour proliferation and metastasis (Guzińska-Ustymowicz et al., 2009; Yu et al., 2013; Zheng et al., 2013). Cell cycle progression is mediated by activating specific cyclin-CDK complexes at different intervals (Ding et al., 2020). In tumour cells, CD1 was dysregulated in cancer cells, including colon cancer (Mermelshtein et al., 2005). In the current study, the overexpressed CD1 was downregulated in response to CaM antagonist treatment (Figure 2b). As CaM is also accumulated at the G1 phase, inhibitory action of CaM by TPZ and TFP could induce cell cycle arrest at G0/G1 by decreasing the cyclin D1 (Feng et al., 2018). This data provide evidence that calmodulin antagonist induces cell cycle arrest by downregulating CD1 and in colon cancer cells.

On the other hand, proliferating cell nuclear antigen (PCNA) expression was also downregulated following PTZ and TFP treatments. PCNA is a nuclear protein and proliferation marker that accumulates in the late G1 and early S phases. It is used as a biomarker that accurately reflects cellular proliferation rates and DNA synthesis (Bologna-Molina et al., 2013). Increased PCNA expression associated with a dysregulation in cell proliferation is strongly related to prognosis and survival in colon cancer (Zhou et al., 2018). We, therefore, hypothesize that PTZ and TFP might regulate cell cycle progression by collectively attenuating CD1 and PCNA activation, thereby suppressing the proliferation of colon cancer cells.

The present study revealed that POPDC1 is downregulated in all types of colon cancer cells, in particular SW480 cells (Figure 3a). Based on accumulated evidence, it has been concluded that the suppression of POPDC1 promotes malignant phenotypes in various cancers (Han et al., 2019). Early study of *POPDC1* expression has shown that the *POPDC1 mRNA* level is downregulated in colon cancer cells (Williams et al., 2011). In agreement with that earlier finding, our data shows that colon cancer cells in types have significantly lower expression of POPDC1 than in a non-cancerous cell. The expression of POPDC1 is reduced substantially at SW480 compared to the malignant types, HCT-15 and COLO205 (Figure 3a).

POPDC1 is an established cell adhesion molecule that serves a role in maintaining epithelial integrity (Osler et al., 2005; Wada et al., 2001). One of the hallmarks of cancer is loss of cell adhesion which allows cells to metastasize (Hanahan and Weinberg, 2011). In hepatocellular carcinoma, POPDC1 loses its expression at

cell junctions, which triggers the Epithelial-Mesenchymal-Transition (EMT) (Han et al., 2014), leading to increase in cell migration. Our findings provide evidence that the reduction of POPDC1 expression enhances proliferation and migration in SW480 colon cancer cells. Cell migration was promoted following POPDC1 gene knockdown (Figure 3d), and the protein levels of CD1 and PCNA were upregulated (Figure 3c), revealing that POPDC1 might regulate these two key molecules that modulate cell proliferation and cell cycle. Interestingly, compared to other studies, the *POPDC1* expression levels are not inversely correlated with the metastatic potential following the colon cancer progression. We believe the role of POPDC1 is in the initiation of metastasis characteristics of cancer cells, namely phenotype changes. The expression of *POPDC1* at early tumorigenesis determines its ability to progress to a metastatic phenotype (Tucker and Zorn, 2021). Hence, we hypothesize that stabilizing POPDC1 expression at the early phase of colon cancer might suppress its metastasis ability.

We further showed that POPDC1 is upregulated in response to treatment with CaM antagonists. Our findings suggest that CaM antagonists mediate inhibition of cell migration and proliferation in colon cancer cells via inducing the localization of POPDC1 to the cell membrane (Figure 4a-c). The downregulation of POPDC1 on the cell membrane is recovered after PTZ and TFP treatment. POPDC1 has a high affinity for cAMP and might also be involved in the cAMP-mediated regulation of cell migration and proliferation (Amunjela and Tucker, 2016; Schindler and Brand, 2016). POPDC1 binds to cAMP and regulates its levels (Brand and Schindler, 2017), as shown in breast cancer, whereby cAMP interacts with POPDC1 and upregulates *POPDC1* expression (Amunjela and Tucker, 2017). CaM, on the flip side, regulates cAMP via Phosphodiesterase (PDE) or Adenyl cyclase (AC) enzymes (Yan et al., 2016). CaM antagonists inhibit cancer cell growth by inducing an elevation in the cAMP levels through PDE inhibition. CaM antagonist tehranolide reduces erythroleukemic cell proliferation via CaM inhibition, following PDE inhibition and consequent cAMP accumulation (Noori and Hassan, 2014). Through our investigation, we believe that CaM antagonists induce the POPDC1 expression while inhibiting CaM, however further investigation is needed to confirm whether this lead to increase of cAMP levels. Hence, we propose a signalling mechanism in which CaM antagonists suppress CaM-induced PCNA and CD1 activation via POPDC1 in colon cancer, thus inhibiting colon cancer cells proliferation and migration (Figure 5). The CaM antagonist/POPDC1 pathway might be and interesting target for colon cancer therapies via the mechanism of stabilizing *POPDC1* expression in the disease to suppress the metastatic potential or later in the disease when *POPDC1* has lost its expression.

The limitation of this study is that we did not provide a direct evidence of cAMP possible involvement in the proposed mechanism. Further investigation is needed to confirm whether increase of cAMP levels mediates CaM antagonists-induced POPDC1 upregulation in colon cancer cells. A direct evidence incorporating increase of cAMP levels and POPDC upregulation using gene knockdown approach can address this question.

In conclusion, our findings show that CaM antagonists, PTZ and TFP can inhibit colon cancer cells proliferation and metastasis via regulating CD1 and PCNA, two critical players in the cell cycle. POPDC1 might be a potential biomarker for colon cancer diagnosis and prognosis, and calmodulin antagonist is a potential regulator that targets POPDC1.

Author Contribution Statement

Conceptualization, F.T. and C.S.; methodology, F.T, C.S.; software, F.T., S.S.; validation, F.T., S.S., and C.S.; formal analysis, F.T., S.S; investigation, F.T.; resources, F.T.; data curation, F.T.; writing—original draft preparation, F.T.; writing—review and editing, F.T., S.S., C.S.; visualization; supervision, C.S., S.S.; project administration, S.S., L.S., C.S.; funding acquisition, C.S, F.A.M.S, M.H.M.S, P.N. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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