Picropodophyllin, an IGF-1 receptor inhibitor, enhances oxaliplatin efficacy in chemoresistant colorectal cancer HCT116 cells by reducing metastatic potential

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Abstract. The insulin-like growth factor receptor (IGF-1R) axis drives cellular growth, survival and chemoresistance in colorectal cancer (CRC) by promoting proliferative signaling, anti-apoptotic effects and epithelial-mesenchymal transition (EMT). Targeting the IGF-1R pathway is therefore a promising strategy, not only for overcoming drug resistance, but also for reducing migration and metastatic behavior related to EMT. The present study aimed to evaluate the potential of picropodophyllin (PPP), a selective IGF-1R inhibitor, to enhance the effects of oxaliplatin (OX) in HCT116 and OX-resistant HCT116-R cells. Cell viability was evaluated using a resazurin-based assay following 48-h combination treatment with OX at its IC_{50} concentrations (HCT116 cells, 53 μM and HCT116-R cells, 324 μM) and PPP (1 μM). Migration was assessed using wound healing assays, with images captured and analyzed at 0 and 48 h. Additionally, immunofluorescence staining was performed to assess E-cadherin and vimentin expression, evaluating epithelial and mesenchymal characteristics. In HCT116-R cells, the combination of OX (53 μM) and PPP significantly reduced cell viability by 0.65-fold compared with OX alone (P=0.0286). Wound healing assays demonstrated that combining PPP with OX (53 and 324 μM) significantly decreased migration, with 0.34-fold and 0.22-fold reductions, respectively (P<0.05). Immunofluorescence staining revealed that this combination also significantly increased E-cadherin expression, by 1.37- and 1.63-fold, respectively (P<0.05), indicating the role of PPP in enhancing epithelial characteristics and reducing EMT-related drug resistance. These findings highlight the potential for combining PPP with OX to enhance the cytotoxic and anti-metastatic effects of OX in chemo-resistant CRC cells, thus offering a promising strategy for overcoming drug resistance and improving patient outcomes in CRC treatment.

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Abbreviations: IGF, insulin-like growth factor; IGF-1R, IGF-1 receptor; PPP, picropodophyllin; OX, oxaliplatin; EMT, epithelial-mesenchymal transition

Key words: colorectal cancer, OX resistance, IGF-1R, picropodophyllin

Introduction

Colorectal cancer (CRC) is the third most common cause of cancer-related mortalities worldwide. The conventional therapies of CRC start with surgical resection followed by adjuvant therapies, such as radiotherapy and chemotherapy (1,2). Oxaliplatin (OX), one of the most used chemotherapeutic agents in CRC treatment, is a platinum-based antineoplastic agent that promotes apoptosis of tumor cells by blocking DNA replication and transcription processes. Empirical evidence indicates that resistance to OX commonly emerges during treatment, thereby undermining the efficacy of therapeutic interventions (3,4). The main causes for the progression of drug resistance depend on regulations of molecular mechanisms such as apoptosis, DNA damage repair, autophagy, drug uptake and epithelial-mesenchymal transition (EMT) (5). EMT is a biological process in which epithelial cells undergo a phenotypic transformation into a mesenchymal state, and this holds critical relevance in cancer progression, as neoplastic cells develop characteristics linked to increased aggressiveness and drug resistance, facilitating their dissemination (6). Previous studies have substantiated the pivotal involvement of several established signaling pathways, including TGF-β, PI3K/AKT, Ras/MAPK, Wnt/β-catenin, NF-κB and Notch, in orchestrating EMT. Thus, it is imperative to direct efforts toward identifying novel agents capable of reducing or inhibiting EMT and its concomitant role in fostering resistance to chemotherapy, including OX-based resistance (7-10). Therefore, one strategy for overcoming the progression of drug resistance is the targeting of EMT.

The insulin-like growth factor (IGF)-1 axis consists of IGF-1, IGF-2, their receptors [mainly IGF-1 receptor (IGF-1R)]

and binding proteins. IGF-1 binds to its receptor, IGF-1R, a receptor tyrosine kinase, which activates several downstream signaling pathways, including the PI3K/AKT, Ras/MAPK, Wnt/β-catenin and NF-κB pathways, all of which are crucial for cell survival, proliferation and EMT (11-13).

The IGF-1 axis serves a pivotal role in promoting EMT by activating key signaling pathways such as PI3K/AKT and MAPK/ERK. The activation of AKT stabilizes transcription factors such as Snail and Slug, which suppress epithelial markers and induce mesenchymal traits, thus enhancing cell survival and migration during EMT. Similarly, ERK activation contributes to EMT by promoting mesenchymal marker expression and further repressing E-cadherin, enhancing invasion and motility. The IGF-1 axis also interacts with pathways such as TGF-β and Wnt/β-catenin, amplifying EMT and driving more aggressive cancer behaviors (14,15). Given its central role in metastasis and therapy resistance, targeting the IGF-1 axis and related pathways offers potential for therapeutic strategies in cancer treatment (16).

The activation of the IGF-1 axis occurs when IGFs stimulate IGF-1R, which is overexpressed in several cancer types, including CRC, promoting cell cycle progression and inhibiting apoptosis (17-19). Several studies have demonstrated elevated IGF-1R expression in colon adenocarcinoma tissues compared with normal mucosa, linking this receptor to key cancer hallmarks, such as proliferation and survival. In the study by Shiratsuchi *et al* (20), 66% of patients with CRC displayed positive IGF-1R expression, which was markedly associated with tumor size and depth of invasion. Zhang *et al* (21) reported that IGF-1R gene expression was markedly higher in CRC samples compared with the adjacent normal tissues, and their analysis revealed a strong association between increased IGF-1R expression levels and key clinicopathological features of CRC, including cancer stage and metastasis. Furthermore, in murine models, overexpression of IGF-1R in HCT116/IGF-1R cells led to highly invasive tumors and distant metastases, such as parental cells (22). These findings indicate that IGF-1R inhibitors hold potential as therapeutic strategies to inhibit cancer progression, metastasis and EMT-related processes, as they target pathways such as PI3K/AKT, Ras/MAPK and Wnt/β-catenin, all of which are key factors in cancer development and drug resistance.

Studies investigating the effects of these inhibitors on colon cancer cells remain relatively limited, although several research efforts have focused on targeting the IGF signaling pathway using IGF-1R antagonists such as NVP-AEW541, BMS-754807, OSI-906 (linsitinib), picropodophyllin (PPP), AMG 479 (ganitumab) and MK-0646 (dalotuzumab). These studies underscore the potential therapeutic value of disrupting IGF-1R signaling in cancer treatment, suggesting that these inhibitors may serve a significant role in enhancing anticancer efficacy by inhibiting tumor growth and overcoming resistance mechanisms (12,23-30). For instance, NVP-AEW541, a small molecule IGF-1R inhibitor, was tested in CRC cell lines, such as SW480 and HT-29. The inhibitor effectively blocked IGF-1R signaling, reducing cancer cell proliferation and inducing apoptosis. Additionally, it increased sensitivity to chemotherapy agents, such as 5-fluorouracil, by disrupting critical survival pathways, namely PI3K/AKT and MAPK (23). Similarly, research by Pennarun *et al* (24,25) reported that inhibiting IGF-1R sensitized colon cancer cells to apoptosis mediated by death receptor 5, enhancing apoptotic signaling and reducing cell survival through inhibition of the PI3K/AKT pathway. Similarly, BMS-754807, a dual IGF-1R/insulin receptor inhibitor, was reported to induce cell cycle arrest and apoptosis in CRC cell lines, such as HCT-116 (26,27). Its combination with cetuximab enhanced anticancer effects, showing greater inhibition of cell growth (26). OSI-906 has also demonstrated strong inhibition of IGF-1R signaling, leading to reduced tumor growth and increased apoptosis in both *in vitro* and *in vivo* models (27). Furthermore, MK-0646 (dalotuzumab) combined with OSI-906 has shown enhanced efficacy in CRC xenograft models by reducing cell proliferation and tumor growth (28).

PPP, a selective inhibitor of IGF-1R, has shown promising anticancer effects in several studies on CRC. In the study by Lee *et al* (29), PPP was tested on HCT-116 CRC cells, where it induced G1 phase cell cycle arrest and triggered apoptosis through reactive oxygen species generation. Additionally, PPP activated the p38 MAPK signaling pathway, contributing to its anticancer effects. Similarly, in the study by Sipos *et al* (30), PPP inhibited IGF-1R, leading to the disruption of toll-like receptor 9 signaling and autophagy, which reduced the survival of HT-29 CRC cells. Moreover, Feng *et al* (12) reported that PPP, whether used as a standalone treatment or in combination with other therapies, blocked IGF-1R signaling, reducing cell viability and inducing apoptosis in colon cancer cells. These findings underscore the potential of PPP as a therapeutic agent for treating CRC by targeting key survival and proliferation pathways.

There is limited literature on enhancing chemotherapy responses through IGF-1R inhibition in chemotherapy-resistant CRC cells. However, a study by Codony-Servat *et al* (31) reported that chemotherapy application increased IGF-1R expression, which was notably elevated in OX-resistant cells. These findings suggest that IGF-1R may serve a critical role in the development of chemotherapy resistance and that targeting IGF-1R could be a potential strategy for enhancing treatment efficacy in resistant CRC cells.

Due to the marked involvement of the IGF axis in both drug resistance and EMT, and in EMT-associated drug resistance, blocking IGF-1 signaling may reduce EMT-based phenotypes and resensitize tumors to therapy. However, there is a scarcity of studies investigating agents targeting IGF-1R to enhance the efficacy of chemotherapy in chemotherapy-resistant cancers (32-34). Therefore, the aim of the present study was to develop a more effective treatment strategy for OX-resistant CRC by combining PPP, an IGF-1R inhibitor, with OX. The rationale for this approach was based on the notable role that the IGF-1 axis serves in cancer progression, metastasis and resistance to chemotherapy, particularly through its involvement in EMT and EMT-associated drug resistance.

Materials and methods

Cell culture. Colorectal carcinoma HCT-116 cells (CCL-247; American Type Culture Collection) and OX-resistant colorectal carcinoma cells (named as HCT116-R), were cultured in DMEM (Cegrogen Biotech GmbH) supplemented with 10% fetal bovine serum (Cegrogen Biotech GmbH),

1% penicillin/streptomycin (Cegrogen Biotech GmbH) and incubated at 5% CO_2 and 37˚C in a humidified incubator. To generate OX-resistant HCT-116-R cells, parental HCT-116 cells were exposed to gradually increasing concentrations of OX (cat. no. O9512; Sigma-Aldrich; Merck KGaA) (1-52 μM), over a 6-month period, established as described in our recent study (10). During the resistance development process, to maintain the balance between proliferation and cell death, cells were cultured in culture media supplemented with gradually increasing doses of OX (1-52 μM) at 5% CO_2 and 37˚C in a humidified incubator. After each OX treatment, the cells were maintained in drug-free culture media for 1-2 days to allow recovery and support the development of resistance. The characterization of resistance was confirmed by the higher IC_{50} value of the resistant cells compared with that of the parental cells, along with an increased expression of mesenchymal marker vimentin in the resistant cells relative to the parental cells as assessed by immunofluorescence staining (10).

Cell viability assay. Resazurin, a cell-permeable blue dye, was utilized as a redox indicator to assess cell viability and evaluate the effects of treatment conditions on the HCT116 and HCT116-R cells (10,35,36). Viable cells with active metabolism reduce resazurin to red fluorescent resorufin through the action of mitochondrial dehydrogenase enzymes (35). To determine cell viability, resazurin salt (cat. no. 14322; Cayman Chemical Company) diluted in phosphate buffered saline (PBS) (Cegrogen Biotech GmbH) was added to the culture medium of cells treated with OX and/or PPP (cat. no. S7668; Selleck Chemicals) (1 μM) for 48 h at 37˚C. The cells were then incubated at 37˚C for 4 h. Fluorescence changes in the medium were measured using a microplate reader (Ex/Em 570/580 nm; Varioskan™ LUX Multimode Microplate Reader; Thermo Fisher Scientific, Inc.). The viability of the control group was set to 100%, and the viability of treated groups was calculated relative to the control.

Determination of OX and PPP concentrations. OX was prepared by dissolving it in saline to a concentration of 5 mg/ml and stored at 4˚C. PPP was dissolved in dimethyl sulfoxide (DMSO) (cat. no. D4540; Sigma-Aldrich; Merck KGaA) to a final concentration of 82 mg/ml, and stored at -80˚C.

In the context of the present study, the combination of PPP and OX involved the determination of the non-toxic maximal concentration of PPP and IC_{50} of OX in both parental and OX-resistant CRC cells. For IC_{50} determination, HCT116 cells were treated with varying concentrations of OX at 3, 6, 12, 24, 48, 96 and 192 μM at 37˚C (33-35). Meanwhile HCT116-R cells were exposed to OX at 100, 200, 300, 400, 500, 600 and 700 μM for 48 h at 37˚C (37-39). To determine the non-toxic highest concentration for PPP, cells were exposed to concentrations of 1, 2, 3, 4, 5, 6 and 7 μM PPP for 48 h at 37˚C (40-42). To assess the effect of PPP on enhancing OX sensitivity, OX and PPP were used as single agents and in combination in both HCT116 and HCT116-R cells. The experimental design involved treating both HCT116 parental and HCT116-R OX-resistant CRC cells with OX and/or PPP at varying concentrations. For the HCT116 parental cells, the treatment groups included a Control (untreated), 53 μM OX alone, 1 μM PP alone, and a combination of 53 μM OX + 1 μM PPP. In the HCT116-R OX-resistant cells, the groups consisted of a Control (untreated), 53 μM OX alone, a combination of 53 μM OX + 1 μM PPP, 1 μM PPP alone, 324 μM OX alone, and a combination of 324 μM OX + 1 μM PPP.

To evaluate the potential of enhancing chemotherapy efficacy through the use of lower doses of OX in the presence of PPP, combination studies were performed in OX-resistant HCT116-R cells, using both the OX IC_{50} value determined for resistant cells (324 μM), and the OX IC_{50} value determined for parental HCT116 cells (53 μM) in combination with PPP (1 μM). The aim of this strategy was to provide insights into the ability of PPP to increase sensitivity to OX, potentially enabling the use of lower chemotherapy doses and thereby reducing treatment-related side effects.

Wound healing analysis. A wound-healing model was used to assess the migratory capacities of HCT116 and HCT116-R cells. Once the seeded cells reached 80-90% confluence in 24-well plates, a scratch was generated in the center of the well using a pipette tip. After washing with PBS, HCT116 and HCT116-R cells were treated with one of the following: 1 μM PPP, OX (53 or 324 μM), or a combination of OX (53 or 324 μM) + PPP (1 μM). Subsequently, the migration of cells on either side of the scratch into the open space was monitored under an inverted light microscope (Axio Vert.A1; Zeiss GmbH) at 0 and 48 h (12). Cells were serum-starved throughout the assay. Wound closure was quantified using Image J Software version 1.53 (National Institutes of Health). The wound area at each time point was measured by manually outlining the scratch boundaries using the freehand selection tool in ImageJ (version 1.54h; National Institutes of Health), and the open wound area was quantified in mm after setting the appropriate scale using the 50 μm scale bar present in the original wound healing images. The percentage of wound closure was calculated using the following formula: % Wound closure=[Wound opening (t0) - Wound opening (tx)*]/Wound opening (t0) x100, where * refers to 48 h.

Immunofluorescence staining for epithelial-mesenchymal markers. Immunofluorescence staining was used to assess the EMT specific E-cadherin (epithelial) and vimentin (mesenchymal) markers on HCT116 and HCT116-R cells. The seeded cells treated with 1 μM PPP, OX (53 or 324 μM), or a combination of OX (53 or 324 μM) + PPP (1 μM) for 48 h at 37˚C. After fixation with 4% paraformaldehyde (Thermo Fisher Scientific, Inc.) at room temperature for 20 min, the cells were permeabilized with 0.1% Triton X100 (Thermo Fisher Scientific, Inc.) solution in 1X PBS for 5 min at room temperature. To prevent non-specific antibody binding, the cells were blocked with 1% bovine serum albumin (BSA) (cat. no. D0050-4090; Cegrogen Biotech) in 1X PBS for 30 min at room temperature. Permeabilized cells were incubated overnight at 4˚C with primary antibodies against E-cadherin (cat. no. 14472S; 1:250; Cell Signaling Technology, Inc.) and vimentin (cat. no. NB300-223; 1:250; Novus Biologicals, LLC; Bio-Techne) in the staining buffer (1% BSA diluted in 1X PBS). The cells were then incubated at room temperature for 1 h with secondary antibodies, including Alexa Fluor™-488-goat anti-mouse (cat. no. ab150171; 1:1,000; Abcam) and Alexa Fluor-647 goat anti-chicken (cat. no. 4408S; 1:1,000; Cell Signaling Technology, Inc.) in PBS. Subsequently, the cells

were washed with 1X PBS and incubated for 10 min at room temperature with nucleus stain DAPI (1:1,000; Thermo Fisher Scientific, Inc.). The stained cells were observed using a confocal microscope (Zeiss, LSM 800 Confocal, USA), and fluorescence intensities were analyzed with the accompanying microscope software [Zen 2.1 (Blue Edition); Zeiss AG].

Statistical analysis. All experiments were performed with ≥3 biological and technical replicates. The collected data was subjected to comprehensive analysis and visualization using GraphPad Prism Version 10.3.1 (Dotmatics). The data are presented as mean ± standard deviation. Statistical significance between two experimental groups was determined using the nonparametric Mann-Whitney U test, whilst multiple comparisons were analyzed using the Mann-Whitney U test followed by Bonferroni's correction. The use of this nonparametric test was justified by assessing data normality using the Shapiro-Wilk test, which confirmed that the data did not follow a normal distribution. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Established concentrations for OX, PPP and combined therapy. The collected data revealed that the IC_{50} values of OX for HCT116 (Fig. 1A) and HCT116-R (Fig. 1B) cells were 53 and 324 μM, respectively. Furthermore, to evaluate the potential alteration of OX efficacy without interference from PPP on cell viability, the highest non-toxic highest concentration of PPP was determined for both HCT116 (Fig. 1C) and HCT116-R cells (Fig. 1D) by applying PPP at a concentration range of 1-7 μM. PPP treatment resulted in a dose-dependent reduction in cell viability in both HCT116 and HCT116-R cells. In HCT116 cells, no significant difference was observed between the control and 1 μM PPP groups ($P>0.999$). However, a significant decrease in cell viability was demonstrated at 2 μM ($P=0.0373$), 3 μM ($P=0.0385$), 4 μM ($P=0.0137$), 5 μM ($P=0.0200$), 6 μM ($P=0.0072$) and 7 μM ($P=0.0018$), compared with the control. Similarly, in HCT116-R cells, 1 μM PPP did not induce a significant change ($P>0.999$), whilst a significant reduction in cell viability was demonstrated at 2 μM ($P=0.049$), 3 μM ($P=0.0060$), 4 μM ($P=0.0127$), 5 μM ($P=0.0012$), 6 μM ($P=0.0012$) and 7 μM ($P=0.0027$). These results suggest a dose-dependent effect of PPP on both HCT116 and HCT116-R cells, with cytotoxicity observed at concentrations of ≥2 μM when each PPP concentration group was compared separately with the control group. Therefore, the highest non-toxic concentration for PPP was determined to be 1 μM, which was used in combination with OX in subsequent experiments.

PPP potentiates OX sensitivity in resistant CRC cells. To assess the effect of PPP (1 μM) on enhancing OX sensitivity, the determined OX IC_{50} value (53 μM) in HCT116 parental cells was used in combination therapy. Additionally, two different combination strategies were applied in OX-resistant HCT116-R cells. First, the OX IC_{50} dose (324 μM) for HCT116-R cells was combined with 1 μM PPP in resistant cells. Second, to assess the effect of 1 μM PPP at lower OX concentrations, the combination of 53 μM OX + 1 μM PPP was also evaluated. The aim of this approach was to provide insight into the potential of using lower doses of OX in the presence of PPP to enhance chemotherapy efficacy, and the findings suggest a treatment strategy that could improve the effectiveness of low-dose chemotherapy whilst reducing side effects.

The combined treatment of 53 μM OX + 1 μM PPP in HCT116 cells was associated with a reduction in cell viability compared with 53 μM OX treatment alone ($P=0.0286$; Fig. 1E). Moreover, treatment of HCT116-R cells with 53 μM OX demonstrated a marked reduction in cell viability compared with the group treated with 53 μM OX alone (0.65-fold decrease; $P=0.029$; Fig. 1F). In HCT116 cells, the combined treatment of 324 μM OX + 1 μM PPP also showed a similar trend of reduced viability compared with 324 μM OX alone, indicating the potential sensitizing effect of 1 μM PPP on OX treatment, although statistical significance was not reached ($P>0.05$; Fig. 1F).

PPP attenuates migration in resistant CRC cells. Wound healing assays were performed to assess the migration of HCT116 and HCT116-R cells. In HCT116 cells, wound closure (migration rate) was 20.4±12.57% in the control group, 23.98±7.11% in the OX (53 μM) group, 10.80±8.30% in the PPP (1 μM) group and 13.89±11.42% in the 53 μM OX + 1 μM PPP group (Fig. 2A and B). The PPP (1 μM) group showed a significant reduction in wound closure compared with that in the control group ($P=0.020$), demonstrating that PPP (1 μM) alone could limit cell migration. In the 53 μM OX + 1 μM PPP group, wound closure was decreased further compared with OX alone, indicating that the combination more effectively impaired migration; however, this reduction was not statistically significant ($P=0.200$).

In HCT116-R cells, wound closure in the control, PPP (1 μM), OX (53 μM), 53 μM OX + 1 μM PPP, OX (324 μM) and 324 μM OX + 1 μM PPP groups were determined to be 21.17±2.21, 10.22±4.49, 35.64±11.74, 12.35±5.37, 27.27±12 and 6.05±2.69%, respectively (Fig. 2C and D). PPP (1 μM) alone significantly reduced migration in comparison with the control group, indicating its effectiveness in limiting migration in resistant cells ($P=0.028$). The combination treatment, 53 μM OX + 1 μM PPP, further significantly decreased the migration rate in comparison with OX (53 μM) alone (0.34-fold decrease; $P=0.029$). Moreover, the combination of 324 μM + 1 μM PPP resulted in a significantly lower wound closure percentage compared with OX (324 μM) alone, highlighting the greater degree of migration reduction achieved using the combination therapy (0.22-fold decrease; $P=0.028$). Overall, the combination of OX (53 μM or 324 μM) and PPP (1 μM) reduced the migration rate of HCT116-R cells compared with OX (52 μM or 324 μM) alone ($P<0.05$), indicating that PPP effectively impairs the migration of OX-resistant cells, even at different concentrations of OX, making it a promising adjunct in overcoming the migration of OX-resistant CRC cells.

PPP preserves the epithelial phenotype against the mesenchymal phenotype in resistant CRC cells. Immunofluorescence staining was performed on HCT116 and HCT116-R cells to evaluate the expression levels of epithelial biomarker E-cadherin and the mesenchymal biomarker vimentin following single and combination treatments.

The fluorescence intensity of E-cadherin in HCT116 cells was significantly increased in the combination of 53 μM OX +

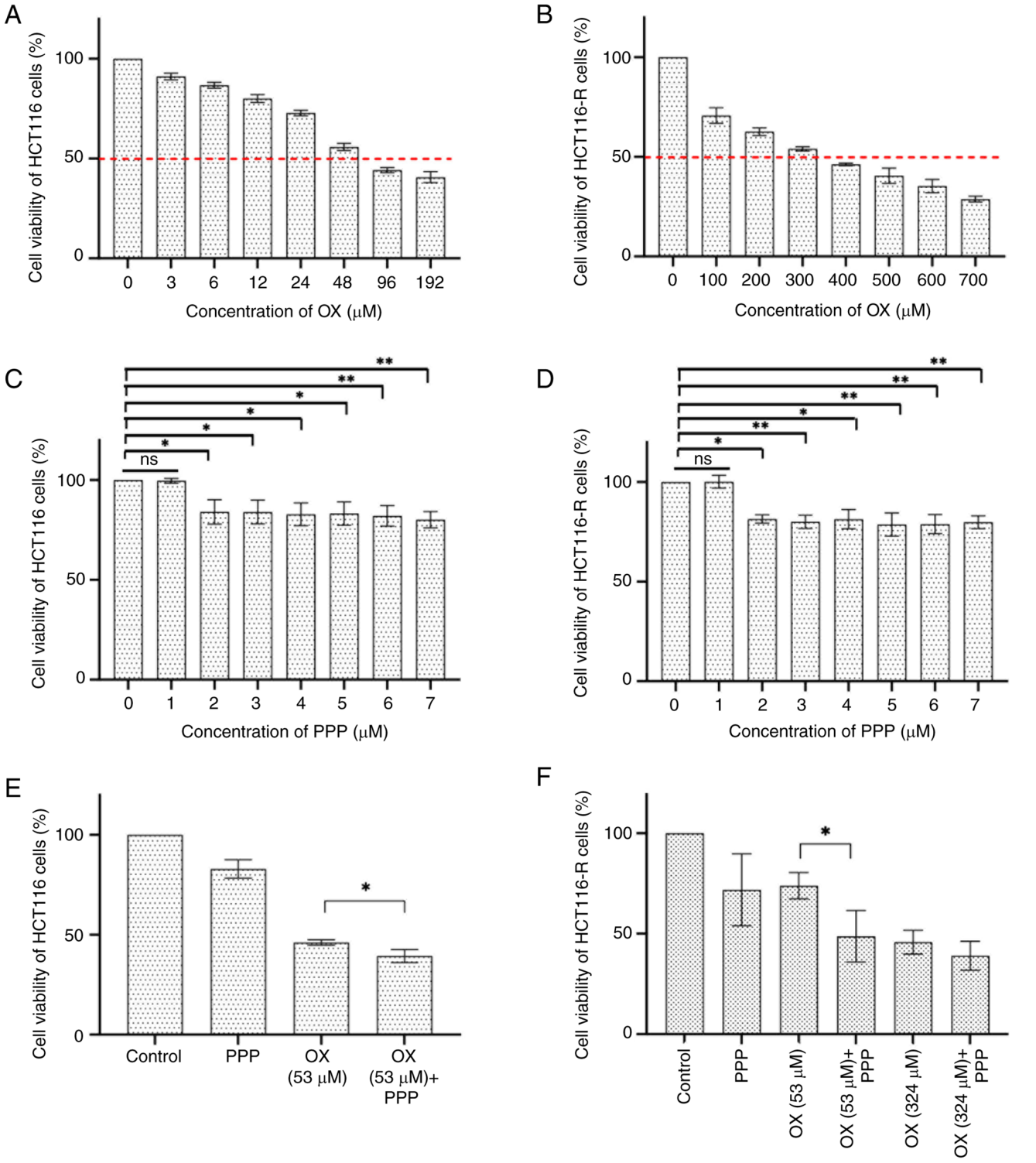

Figure 1. Effects of single and combined treatments with OX and PPP on the viability of HCT116 and HCT116-R cells. IC_{50} value determination of OX for (A) HCT116 cells and (B) HCT116-R cells. Cell viability assay results for (C) HCT116 and (D) HCT116-R cells treated with varying concentrations of PPP (1-7 μM). Cell viability of (E) HCT116 and (F) HCT116-R cells following single and combined treatments with OX and PPP. *P<0.05, **P<0.01. OX, oxaliplatin; PPP, picropodophyllin; -R, OX-resistant; ns, not significant.

1 μM PPP group compared with that in the OX (53 μM) group alone (P<0.001), suggesting a synergistic effect of the combination treatment in promoting epithelial characteristics. The PPP (1 μM) group also demonstrated significantly increased fluorescence intensity for E-cadherin expression compared with the control (P<0.001) (Fig. 3A and B). Furthermore, the combination of 53 μM OX + 1 μM PPP revealed a slight but statistically insignificant increase in the fluorescence intensity of vimentin levels compared with OX (53 μM) treatment alone (P>0.05). Treatment with PPP (1 μM) also slightly increased vimentin levels compared with the control, but the change was not statistically significant (P>0.05) (Fig. 3C).

In HCT116-R cells, the 53 μM OX + 1 μM PPP group demonstrated a statistically significant increase in E-cadherin

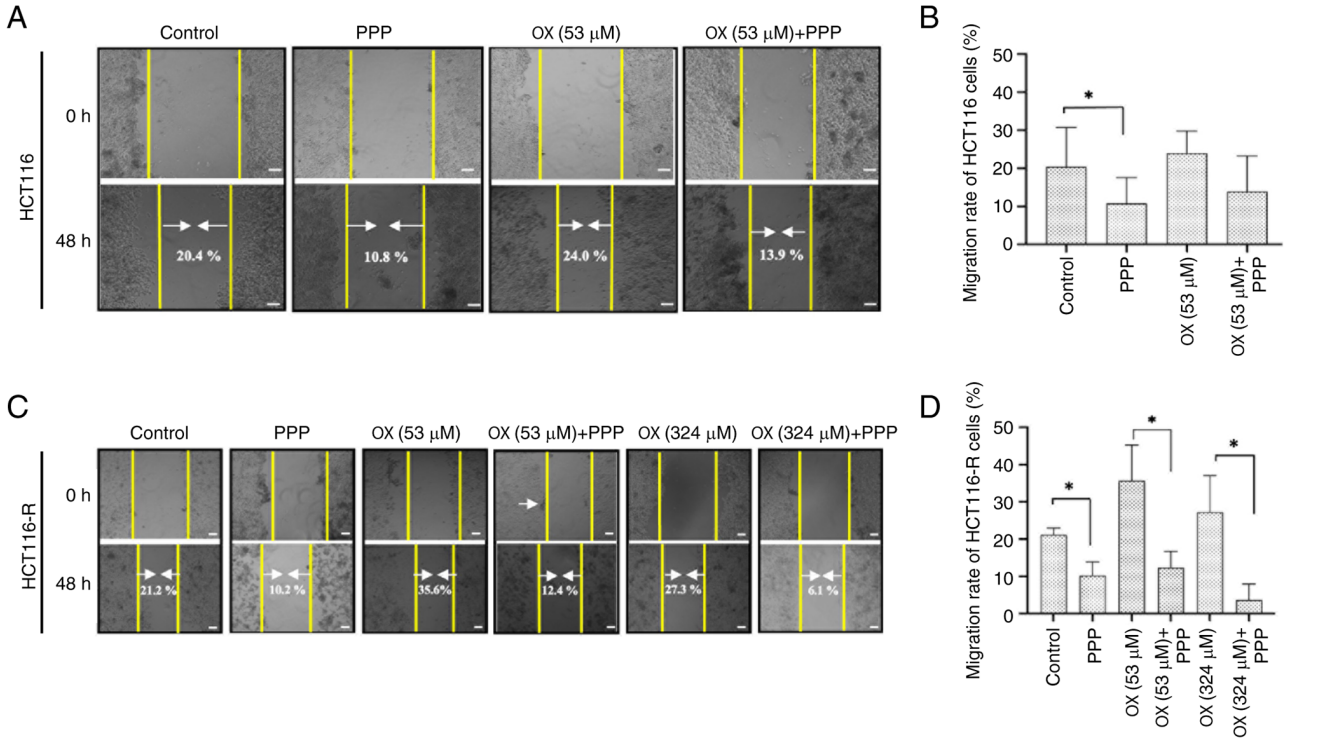

Figure 2. Evaluation of migration of HCT116 and HCT116-R cells using wound-healing assays. (A) Representative images showing wound closure at 0 and 48 h in HCT116 cells. Scale bar, 50 μm. (B) Quantitative analysis of wound closure (migration rate) in HCT116 cells, performed to assess the effects of single and combined treatments of OX and PPP. (C) Representative images showing wound closure at 0 and 48 h in HCT116-R cells. Scale bar. 50 μm. (D) Quantitative analysis of wound closure (migration rate) in HCT116-R cells, performed to evaluate the effects of single and combined treatments of OX and PPP treatments. *P<0.05. OX, oxaliplatin; PPP, picropodophyllin; -R, OX-resistant.

levels compared with the OX (53 μM) group alone (1.37-fold increase; P=0.0104). This indicates the ability of PPP to enhance epithelial marker expression in resistant cells even with low doses of OX (Fig. 3D and E). The 324 μM OX + 1 μM PPP group also demonstrated a significant increase in E-cadherin expression compared with the OX (324 μM) group alone (1.63-fold increase; P=0.001). This result suggests that PPP (1 μM) can enhance the ability of OX to induce epithelial features in resistant cells at a higher dose of OX. The PPP (1 μM) group also revealed significantly increased fluorescence intensity for E-cadherin expression compared with the control group (P<0.001). However, the fluorescence intensity of vimentin in HCT116-R cells demonstrated no significant differences between the following groups: Control and PPP; OX (53 μM) and 53 μM OX + 1 μM PPP; and OX (324 μM) and 324 μM OX + 1 μM PPP (P>0.05). The expression levels of vimentin remained consistent across all treatment groups, suggesting that PPP does not affect its expression in the presence or absence of OX in HCT116-R cells (Fig. 3F).

The aforementioned findings suggest that PPP induces an enhancement in epithelial characteristics in both HCT116 and HCT116-R cells and supports the potential use of PPP (1 μM) to improve the efficacy of OX in CRC treatment.

Discussion

Despite advancements in novel treatments, OX-based chemotherapy combinations continue to serve a key role in CRC treatment. The major issue in the OX treatment of CRC is acquired resistance. The IGF-1R signaling axis serves a critical role in the proliferation and growth of tumor cells in an EMT-based metastatic phenotype. Different studies have reported that IGF-1 production and IGF-1R expression can inhibit cell death and cause drug resistance by upregulating survival protein in cancers. As EMT serves a crucial role in acquired resistance, there is an increasing trend towards agents that increase treatment sensitivity by targeting EMT inhibition (16,43-45). In line with this, the present study aimed to propose a novel combination strategy using an IGF-1R inhibitor with the aspect of proliferation, drug resistance and EMT-supportive IGF-1R signaling, to increase the chemotherapeutic efficacy of OX-resistant CRC. Based on the results, the present study aimed to propose a more effective treatment strategy by using PPP in combination with OX to reduce the proliferative and EMT-based aggressive phenotype of OX-resistant CRC cells. We hypothesized that, by targeting IGF-1R, PPP may inhibit the proliferation and EMT-driven aggressiveness of OX-resistant CRC cells, thus re-sensitizing the tumors to chemotherapy and enhancing treatment efficacy. This dual approach seeks to overcome chemotherapy resistance, which is a major obstacle in CRC treatment, by attacking both survival and drug resistance pathways.

IGF-1R proteins have emerged as important targets for cancer therapy. Previous studies have been performed with IGF-1R-targeted agents such as NVP-AEW541, OSI-906 (linsitinib), PPP, AMG 479 (ganitumab) and MK-0646 (dalotuzumab) in several human cancers, showing that these

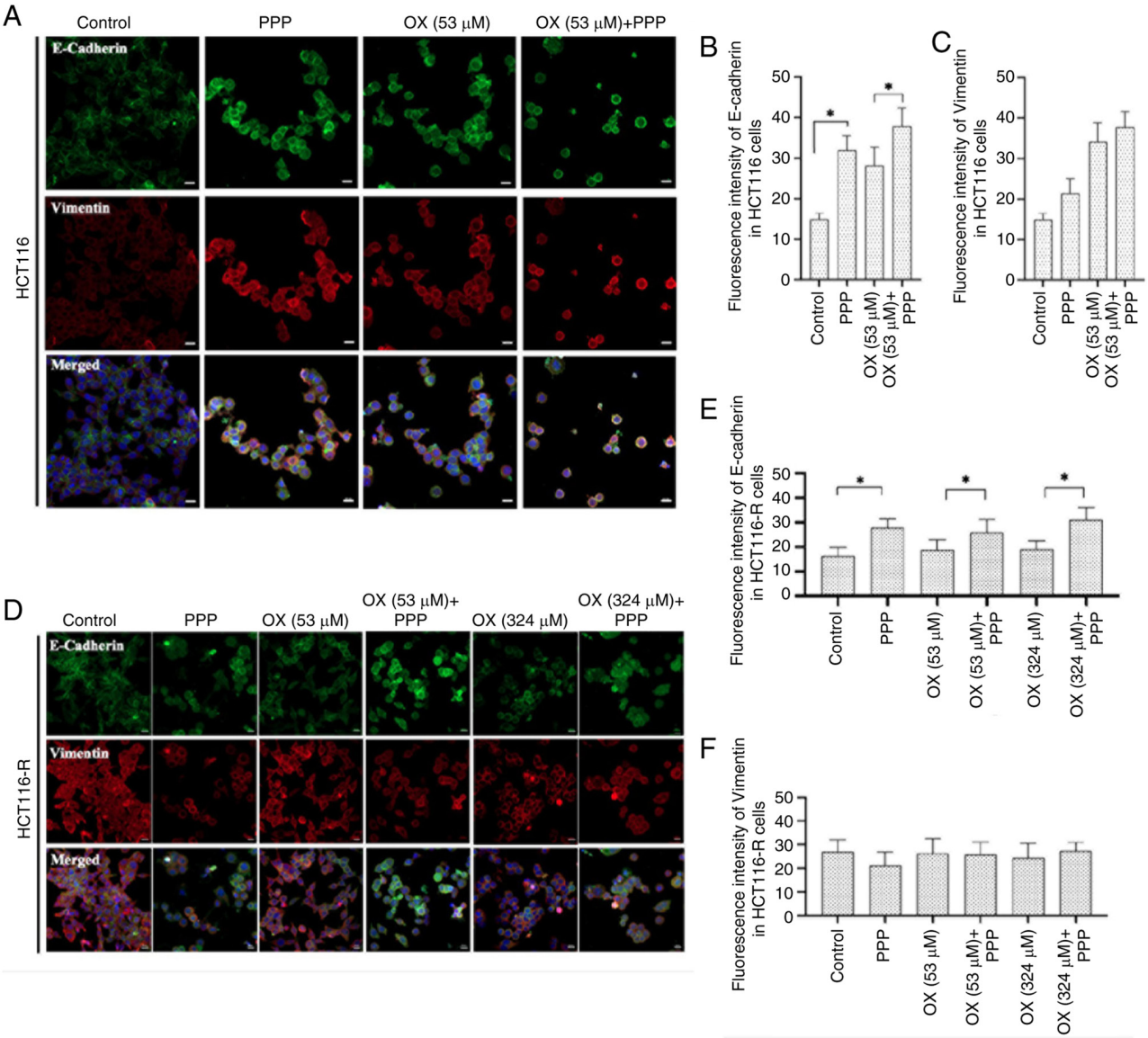

Figure 3. Evaluation of the expression levels of the epithelial marker E-cadherin and the mesenchymal marker vimentin in HCT116 and HCT116-R cells following single and combination treatments with OX and PPP. (A) Representative fluorescence images showing E-cadherin and vimentin expression in HCT116 cells. Scale bar, 20 μm. Quantitative analysis of (B) E-cadherin and (C) vimentin fluorescence intensity in HCT116 cells. (D) Representative fluorescence images showing E-cadherin and vimentin expression in HCT116-R cells. Scale bar, 20 μm. Quantitative analysis of (E) E-cadherin and (F) vimentin fluorescence intensity in HCT116-R cells. *P<0.05. OX, oxaliplatin; PPP, picropodophyllin; -R, OX-resistant.

compounds can inhibit tumor cell growth and induce apoptosis (12,23-30). However, the substantial role of the IGF axis in both drug resistance and the EMT has led to a notable lack of studies exploring agents that target the IGF-1R to augment the effectiveness of chemotherapy in chemotherapy-resistant cancers (32-34,46). Therefore, the present study specifically focused on assessing the impact of PPP in combination with OX to enhance drug sensitivity and attenuate the drug resistance-promoting EMT phenotype in OX-resistant CRC cells.

Initially, the aim of the present study was to determine the highest non-toxic dose of PPP to prevent any PPP-induced cytotoxicity when utilized in combination with OX, forming part of the combination therapeutic strategy. Feng *et al* (12) investigated the anticancer effect of PPP in colon carcinoma cell lines and reported a net reduction in viable cells when treated with PPP at concentrations ≥1 μM. Another study by Wang *et al* (41) investigated the therapeutic response of PPP in colorectal carcinoma, and reported that PPP markedly suppressed the growth of CRC cells at a concentration of 1 μM. The analysis in the present study demonstrated that 2 μM PPP affected cell viability, therefore 1 μM PPP was considered as the highest possible concentration that had no cytotoxic effect on CRC cell lines. Furthermore, the present study used the maximum non-toxic concentration of PPP in combination with OX to enhance OX sensitivity and overcome OX-resistance. In the present study, cytotoxic effects were observed at concentrations >1 μM which is consistent with previous studies that reported a reduction in cell viability at concentrations ≥1 μM (12,14). These findings suggested that PPP begins to exhibit cytotoxic effects at concentrations ≥1 μM.

Although OX is a standard treatment option in CRC, it leads to toxicity (especially peripheral neurotoxicity), a serious

dose-limiting problem in the clinic. Due to the OX-related side effects and toxicities, the treatment may need to be interrupted before remission can be achieved (47). Therefore, the dosage should be decreased for OX to prevent other toxicities in healthy tissue, but this decrease will affect other parameters, the most crucial being the treatment capacity. Therefore, PPP could be used as an adjuvant agent at its nontoxic concentration to enhance the treatment effect of OX and provide high efficacy in low dosages by avoiding OX-related healthy tissue toxicities in patients with CRC.

To evaluate the impact of PPP on enhancing sensitivity to OX in CRC, the present study used the IC_{50} value of OX (53 μM) in HCT116 parental cells for combination therapy. In OX-resistant HCT116-R cells, two different combination strategies were tested. First, the IC_{50} dose of OX for resistant cells (324 μM) was combined with PPP (1 μM) to assess the potential of overcoming resistance. Second, to assess the effect of PPP (1 μM) at lower OX concentrations, OX was tested at the lower parental IC_{50} value (53 μM) in combination with PPP (1 μM). This latter approach aimed to explore whether PPP (1 μM) could enhance OX efficacy at lower drug concentrations, potentially improving treatment outcomes while minimizing side effects. The results of this strategy offer a promising avenue for reducing chemotherapy dosages, minimizing toxicity, whilst maintaining or enhancing therapeutic efficacy in resistant cancer cells.

The results of the present study demonstrate that PPP significantly enhances the cytotoxic effects of OX in both HCT116 and OX-resistant HCT116-R CRC cells. In non-resistant HCT116 cells, the combination of 53 μM OX + 1 μM PPP led to a statistically significant decrease in cell viability compared with OX alone. Similarly, in resistant HCT116-R cells, the same combination further reduced cell viability, indicating that PPP improves sensitivity to OX. Whilst the higher concentration of OX (324 μM) combined with PPP (1 μM) showed a trend toward greater cytotoxicity in resistant cells, this difference was not statistically significant. Overall, PPP appeared to enhance the effectiveness of OX, particularly at lower doses.

The results of the current study, namely, that PPP enhances the cytotoxic effects of OX, are consistent with prior research highlighting the therapeutic potential of PPP. PPP has been widely recognized for its role as a selective IGF-1R inhibitor, effectively disrupting cancer cell survival pathways, such as the PI3K/AKT and MAPK/ERK pathways, which are often upregulated in chemotherapy-resistant cancer cells. Feng *et al* (12) demonstrated that PPP effectively inhibits IGF-1R signaling, leading to reduced viability and increased apoptosis in colon cancer cells, both as a standalone treatment and in combination with other therapies. Similar to the findings of the present study, Lee *et al* (29) reported that PPP enhances the anticancer effects of chemotherapeutic agents by inducing apoptosis and inhibiting cell proliferation in CRC cells, further supporting the synergistic effect observed in the combination of OX and PPP. Moreover, the finding that PPP increases OX sensitivity at both lower (53 μM) and higher (324 μM) concentrations aligns with study by Sipos *et al* (30) showing that PPP can overcome drug resistance mechanisms, particularly in cells exhibiting an EMT phenotype, which is closely associated with chemotherapy resistance. The previous study demonstrated that PPP disrupts EMT signaling and autophagy, reducing the survival of chemo-resistant CRC cells, further supporting the potential role of PPP in combination therapies targeting drug-resistant cancers.

To the best of our knowledge, there are no studies specifically addressing the combination of PPP with chemotherapy in CRC, but a limited number of studies focus on combination therapies involving PPP in other types of cancer. Tarnowski *et al* (40) evaluated the effects of PPP in combination with actinomycin-D and cisplatin in rhabdomyosarcoma cells and demonstrated that PPP increased the sensitivity to chemotherapy. Moreover, in another study by Duan *et al* (48), PPP was reported to enhance the cytotoxic effects of doxorubicin in osteosarcoma cell lines that had developed resistance to doxorubicin. In addition, downregulation of IGF-1R expression in drug-resistant cell lines by small interfering RNA led to the restoration of sensitivity to doxorubicin (48). Therefore, PPP holds promise as a potent adjunct to OX in treating chemo-resistant CRC, potentially improving patient outcomes by sensitizing tumors to lower doses of chemotherapy, reducing both drug toxicity and resistance (27,49).

The wound healing assays in the present study demonstrated that PPP effectively reduces the migration of both HCT116 and OX-resistant HCT116-R CRC cells. The findings, demonstrating that PPP was associated with a significant reduction in the migration of both types of CRC cells, align with earlier research that highlights the ability of PPP to impair cancer cell migration and metastasis. For example, Feng *et al* (12) reported that PPP reduced the migration of HCT116 cells in a dose-dependent manner. This is consistent with the results of the present study, where PPP reduced migration in combination with OX, especially in resistant cells. Additionally, whilst the combination of PPP and OX (53 μM) led to a reduction in migration in HCT116 cells compared with OX alone, this decrease did not reach statistical significance. By contrast, the combination of PPP with either low-dose OX (53 μM) or high-dose OX (324 μM) led to a statistically significant reduction in migration in HCT116-R cells, compared with OX alone ($P<0.05$). This suggests that PPP not only enhances the cytotoxic effects of OX, but also significantly reduces the migratory potential of OX-resistant CRC cells, making it a promising adjunct for overcoming their migratory capacity. The observed differences in the effects of the combinatorial use of PPP with OX on HCT116 and HCT116-R cells may be attributed to two main factors. Firstly, the wound healing assay used in the present study may lack the sensitivity to detect subtle changes in migration, particularly in non-resistant HCT116 cells, where the migration rate might not be significantly altered by PPP and OX under these specific experimental conditions. Secondly, biological mechanisms likely serve a role in these differences. Non-resistant HCT116 cells may lack EMT-related changes, which are commonly associated with increased migration and invasiveness (50). By contrast, HCT116-R cells, which are resistant to OX, exhibit significant differences in migration, suggesting the involvement of EMT or other resistance-associated mechanisms in driving migration. These findings highlight the distinct biological and functional characteristics of resistant and non-resistant cell populations and underscore the potential of PPP in targeting migration in chemo-resistant cancer cells.

The paradoxical finding that OX alone increased the migration of resistant cells, potentially through mechanisms related to EMT, is supported by previous studies. EMT is often linked to increased cell migration and metastasis, particularly in chemo-resistant cancers, where it enhances the invasiveness of tumor cells (50-52). Previous studies have demonstrated that IGF-1R inhibition, as achieved through PPP, can suppress EMT and reduce the metastatic potential of cancer cells (29). This reversal of OX-induced migration by PPP in resistant cells further underscores its potential role as an adjunct therapy to prevent metastasis-related phenotypes in OX-resistant CRC. Moreover, similar findings from Sipos *et al* (30) reinforce the hypothesis that PPP can disrupt EMT processes by targeting IGF-1R signaling, thereby reducing drug resistance and metastatic behavior in cancer cells. By targeting these pathways, PPP not only enhances the cytotoxicity of OX but also limits the metastatic potential, making it a promising approach for overcoming the dual challenges of drug resistance and metastasis in CRC treatment.

The immunofluorescence results of the present study, which demonstrated that PPP preserved the epithelial phenotype in both HCT116 and OX-resistant HCT116-R CRC cells by promoting E-cadherin expression, align with prior research that highlights the ability of PPP to influence EMT markers. In the present study, PPP was demonstrated to significantly increase E-cadherin levels, an important epithelial marker, without significantly affecting the mesenchymal marker vimentin, particularly when combined with OX. These findings are consistent with reports by Lee *et al* (29) and Feng *et al* (12), who reported that PPP not only induces apoptosis, but also inhibits key EMT markers, thereby promoting epithelial traits in cancer cells.

Previous studies have also reported that EMT is a critical mechanism by which cancer cells acquire drug resistance and increased metastatic potential (14). Sipos *et al* (30) reported that targeting the IGF-1R pathway with PPP disrupted EMT signaling, reinforcing epithelial characteristics such as E-cadherin expression, which corresponds with the findings of the present study. The ability of PPP to promote E-cadherin expression in both non-resistant and resistant cell lines suggests that it could serve a vital role in combating chemoresistance by inhibiting EMT, a key process involved in drug resistance and metastasis. Additionally, Zhang *et al* (21) reported that IGF-1R serves a pivotal role in maintaining EMT traits, contributing to the aggressive and resistant nature of CRC cells. The findings of the present study, where PPP enhances E-cadherin expression without significantly affecting vimentin, further support the notion that targeting IGF-1R with PPP could potentially reverse EMT-associated resistance and enhance the effectiveness of chemotherapy. In congruence with these outcomes, Li *et al* (16,46) aimed to enhance the drug sensitivity of epidermal growth factor receptor-tyrosine kinase inhibitor-resistant non-small cell lung cancer cells through the inhibition of insulin-such as growth factor 1 receptor via PPP. The study reported a diminution in mesenchymal markers including Snail, Slug, zinc finger E-box binding homeobox 1 and Vimentin, and an elevation in the epithelial marker E-cadherin following treatment with PPP.

The combination treatment of 53 μM OX + and 1 μM PPP in parental HCT116 cells resulted in a slight, statistically non-significant increase in vimentin fluorescence intensity compared with OX treatment alone, whilst PPP (1 μM) alone also led to a modest, non-significant increase relative to the control. These findings may reflect inherent biological variability and the limitations of a small sample size, potentially contributing to the lack of statistical significance. Additionally, it is possible that PPP may interact with alternative signaling pathways or compensatory mechanisms, such as stress responses or overlapping EMT pathways (such as, TGF-β/Smad and Wnt/β-catenin), which could sustain or slightly enhance vimentin expression. Moreover, specific genetic or epigenetic characteristics of the HCT116 cell line may influence vimentin expression, making it less responsive to PPP. In HCT116-R cells, vimentin fluorescence intensity did not demonstrate significant changes across treatment groups, including PPP, OX and their combinations. This lack of response may indicate the possibility of intrinsic resistance in HCT116-R cells, potentially involving a stabilized EMT phenotype or constitutive activation of EMT pathways. Additionally, the doses of PPP and OX used may not have been optimal for modulating vimentin expression, and cellular heterogeneity or other regulatory mechanisms could contribute to the observed outcomes. In summary, the preservation of epithelial markers such as E-cadherin and the lack of significant changes in mesenchymal traits such as vimentin upon PPP treatment, particularly in combination with OX, suggest that PPP could be an influential adjunct to chemotherapy by mitigating EMT-associated drug resistance in CRC.

In concordance with analogous results from the literature, the data from the present study indicate that PPP markedly augments therapeutic efficacy in CRC by potentially counteracting mechanisms of drug resistance. This underscores the need for an innovative dual therapy strategy for the management of OX-resistant CRC. The use of PPP in the treatment protocol is an innovative strategy aimed at increasing the chemotherapy efficacy of OX. This strategy aims to reverse the mesenchymal properties of cancer cells by sensitizing them to OX. The proposed dual therapy not only addresses the OX resistance in CRC but also offers a promising way to improve the overall efficacy of chemotherapy in this context.

However, the present study has several limitations that need to be addressed in future research. First, the experiments were performed solely on *in vitro* models using two CRC cell lines, HCT116 and OX-resistant HCT116-R. This approach may not fully capture the complexity and heterogeneity of tumors in *in vivo* conditions. Moreover, there was no attempt to account for the tumor microenvironment, which can notably influence drug resistance and response (53,54). Additionally, there is a need to further explore the long-term effects of PPP on cell behavior and the molecular mechanisms underlying its action, particularly regarding EMT and IGF-1R signaling. Furthermore, vimentin may not be a primary marker affected by PPP, which could instead predominantly target other mesenchymal markers such as N-cadherin. Evaluating additional EMT markers and increasing sample sizes are crucial to better understand the broader effects of PPP on EMT regulation. Future studies should include IGF-1R knockdown and overexpression experiments to improve the understanding of the role of this pathway in OX resistance. These experiments could provide

deeper insights into how IGF-1R signaling influences cancer cell survival, migration and EMT. Additionally, future studies should include *in vivo* models and a broader range of CRC subtypes to better understand the efficacy of PPP in overcoming OX resistance. Detailed mechanistic studies are also needed to provide a clearer picture of how PPP modulates cancer cell survival, migration and resistance pathways.

In summary, PPP has emerged as a promising adjunctive agent to enhance the therapeutic efficacy of OX in CRC. The present study highlights potential avenues for future research, particularly in exploring novel treatment modalities. Specifically, integrating EMT suppressive agents with conventional chemotherapeutic approaches could further improve treatment effectiveness. However, due to the complex and multifactorial nature of CRC, numerous therapeutic targets remain to be explored for optimizing therapeutic outcomes. Therefore, it is essential to identify next-generation drug targets and refine current treatment protocols to develop comprehensive therapeutic strategies aligned that address the intricate pathogenesis of CRC.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

NK, YB and GCK contributed to the conceptualization and design of the study. NK, HK, TS and GCK were responsible for data acquisition and analysis, while NK, HK, TS, YB and GCK contributed to the interpretation of the data. All authors were involved in drafting the manuscript and critically reviewing the manuscript for intellectual content. All authors have reviewed and approved the final version of the manuscript and agree to be accountable for the accuracy and integrity of the work. NK, YB and GCK confirm the authenticity of all raw data.

Ethics approval and consent to participate

The present research, performed as a Master of Science thesis, was approved by the Dokuz Eylül University Non-Interventional Research Ethics Committee (14.07.2021; approval no. 2021/21-13). Obtaining ethical approval is mandatory for all thesis studies performed under the Dokuz Eylül University Institute of Health Sciences. The present study did not involve the use of human or animal materials, and all necessary institutional permissions and ethical committee approvals were obtained.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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