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Effect of combination treatment of protocatechuic acid with 5-fluorouracil and oxaliplatin on colon cancer Caco-2 cell line

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Among the most common antitumor drugs used in the treatment of colon cancer are 5-fluorouracil and oxaliplatin (5-FU and OXA). However, both these drugs have many side effects, and hence there is a need for new treatment\approach to reduce the side effects aas well as drug concentration. In this context, here, we investigated the effect of addition of protocatechuic acid (PCA) onto either monotherapies or combination therapies of 5-FU and OXA on the human colon cancer (Caco-2) cell line. In addition, we did evaluate the synergistic effect of PCA with 5-FU and OXA. Further, we determined the suppressive effects of different doses of PCA alone or in combination with 5-FU/OXA on cell proliferation after 24 and 48 hours. We identified a suppressive effect of PCA on cell viability at 48 h starting from the dose of 50 µM Matrix metalloproteinase-2 (MMP-2) and MMP-9 gene expression levels and apoptotic effects showed significant increases and decreases depending on the dose and time applied in the experimental groups. The highest synergistic activity was seen at 2:1 concentration of 5-FU+ PCA. Our findings indicate the presence of the cytotoxic and apoptotic effects of PCA in Caco-2 cells at 48 h, increasing with a dose- and time-dependent manner.

Keywords: Anticancer activity, Caco-2, Combination index, Dose reduction index (DRI), Matrix metalloproteinases (MMP)

Colon cancer is globally the third most common cancer, constituting almost 10% of all cancers[1,2]. Although diagnosis and treatment have improved over the years, approximately 50-60% of patients diagnosed with colon cancer develop distant metastases, resulting in severe morbidity and mortality[3]. In fact, currently available chemo-therapeutic agents failed to provide a complete cure or prevent disease recurrence[4]. 5-FU and OXA are among the drugs used in treatment[5,6]. Combined chemotherapy yields more effective outcomes than a single drug does. However, 5-FU and OXA are known to also damage healthy cells, causing many adverse effects. Therefore, new treatments are needed to reduce the drug concentration and side effects of 5-FU and OXA[7].

The plants with antioxidant properties in cancer treatment continue to be successfully used worldwide due to the side effects of drugs[8,9]. Protocatechuic acid (PCA) is a phenolic compound found in various medicinal plants, such as *Hypericum perforatum* L., *Hibiscus sabdariffa* L. and *Ginkgo biloba* L. etc.[10]. It is reported to exert a broad spectrum of pharmacological activities, including antioxidant, antitumor, anticancer, antibacterial, antiallergenic, antidiabetic, antiapoptosis, analgesic, antineoplastic and anti-inflammatory effect[10,11]. PCA has been suggested to be used in combination with various anticancer drugs to increase its therapeutic effects[12]. Despite research with PCA in lung, breast, liver, and ovarian cancer, studies on colon cancer cell lines are limited[13,14]. One of the key molecules involved in tumor invasion and metastasis is extracellular matrix (ESM) elements. ESM acts as a primary barrier to prevent tumor tissue growth and tumor cell spread[15]. Matrix metalloproteinase (MMP)-2 and MMP-9 enzymes, which play a role in ESM regulation, break down ESM and cause destruction of the basal lamina[16]. Cancer cells, on the other hand, gain invasive and metastatic features by overcoming the ESM barrier by elevating these two enzymes[17]. Studies have shown increased activity levels of MMP-2 and MMP-9 in colon cancer patients[18,19].

In this experimental study, we aimed to investigate the effect of the addition of PCA onto either

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monotherapies or combination therapies of 5-FU and OXA in the viability of human colon cancer (Caco-2) cell line.

Materials and Methods

Drugs and Chemicals

PCA, 5-FU and OXA was purchased from Sigma Aldrich (St. Louis, MO).

Cell culture

We obtained a human Caco-2 cell line from Culture Collection of Animal Cells, Foot and Mouth Disease (ŞAP) Institute, Ankara, Turkey. We conducted the study in Alanya Alaaddin Keykubat University Faculty of Medicine Cell Culture Laboratory. Caco-2 cells were cultured in DMEM medium containing 10% fetal bovine serum, 1% penicillin-streptomycin, L-glutamine, and $NaHCO_3$ at 37°C in an environment containing 5% CO_2 and atmospheric humidit[20].

Experimental groups

We determined eight groups in this study as follows: Group I (Control): No chemicals to Caco-2 cells; Group II (PCA): Cells incubated with PCA (25-50-100-250-500-1000-2000 µM) for 24 and 48 h; Group III (5-FU): Cells incubated with 5-FU (5-10-25-50-100-250-500-1000-2000-4000 µM) for 24 and 48 h; Group IV (OXA): Cells incubated with OXA (10-50-100-150-250-500 µM) for 24 and 48 h; Group V (5-FU + OXA): Cells incubated with 5-FU (50 µM) and OXA (50 µM) for 24 and 48 h; Group VI (5-FU + PCA): Cells incubated with 5-FU (50 µM) and PCA (100-250 µM) for 24 and 48 h; Group VII (OXA + PCA): Cells incubated with OXA (50 µM) and PCA (100-250 µM) for 24 and 48 h; andGroup VIII (5-FU + OXA + PCA): Cells incubated with 5-FU (50 µM), OXA (50 µM), and PCA (100-250 µM) for 24 and 48 h.

Cell viability assay

We measured cell viability via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay[21]. Caco-2 cells (1×10^4 cells/well) were seeded in 96-well plates (Grenier Bio-one, Victoria, Australia) and incubated at 37°C in 5% CO_2 for 24 h. Then, cells were incubated with PCA, 5-FU, Oxa, 5-FU+OXA, PCA+OXA, PCA+5-FU, and 5-FU+OXA+ PCA for 24 and 48 h, while control cells were treated with the same volume of culture medium. For PCA, ethanol was used as solvent control and incubated for the same duration. After treatment, a 10 µL aliquot of MTT solution [5 mg/mL in phosphate-buffered saline; Sigma] was supplemented into each well for an additional 3 h incubation. The supernatant was then discarded, and 100 µL dimethyl sulfoxide was added to dissolve the formazan crystals. Plates were placed on a shaking incubator for 15 min, and optical density was measured in an automatic multiplate reader (Synergy™ H1, Biotek, USA) at 570 and 630 nm wavelengths. Each treatment was tested with 6 samples (n=6) and the whole experiment was repeated thrice. Data were expressed as the number of viable cells compared with the percentage of control cells treated with DMEM.

Synergistic effect analysis

The Chou Talalay equation and CompuSyn software (ComboSyn, NJ, USA) were used to determine the combination index (CI) and dose reduction index (DRI). The CI was used to determine the types of drug interactions where CI = 1 indicates additive effect CI <1 indicates synergistic effect and CI >1 represents antagonistic effect[22].

Detection of apoptosis

The possible apoptotic effects of single, double, and triple combined doses of drugs on Caco-2 cells were determined using the Cell Death Detection Elisa PLUS kit (Roche) per the manufacturer's protocol. This assay determines apoptosis by measuring mono- and oligonucleosomes in the lysates of apoptotic cells. The cell lysates were placed into a streptavidin-coated microplate and incubated with a mixture of anti-histone-biotin and anti-DNA-peroxidase. The amount of peroxidase retained in the immunocomplex was photometrically determined with ABTS (3-ethylbenzothiazoline-6-sulfonic acid) as the substrate. Absorbance was measured at ELISA (multiplate reader) at 450-500 nm (Synergy™ H1, Biotek, USA). Three wells were made for each sample. The enrichment factor ratio was calculated by dividing the average absorbance values of the samples by the average absorbance value obtained from the negative control cells.

Real-time PCR

Total RNA was isolated from cells using the Total RNA Purification Isolation Kit (Jena Bioscience, Germany). The purity of isolated RNAs was measured with Eliza Plate Reader (260/280 nm= 1.8-2.1). cDNA was synthesized using the VitaScript™ FirstStrand Reverse Transcription System (Procomcure Biotech GmbH, Austria). We used 10 µg of total RNA in reverse transcription reaction. Obtained

cDNAs were stored for a short time at –20°C for a real-time PCR reaction. Transcription of genes was determined by qPCR (LightCycler®96 Instrument - Roche Diagnostics) using SYBR-Green Master Mix (A.B.T™ 2X, Turkey) with the following PCR conditions: 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. β-actin was used as a housekeeping gene for quantification. Relative mRNA copies were compared with negative control using the comparative cycle threshold method $2^{-\Delta\Delta Ct}$[23].

Statistical analyses

We used SPSS 20.0 for Windows software to analyze study data. The continuous variables were presented as means and standard deviation. For comparisons, we used one-way Annova-Tukey test. Other statistical analyses were calculated using GraphPad Prism (Version 7.04 for Windows, GraphPad Software, USA) software. We used an overall Type-I error level of 5% to infer statistical significance.

Results

Cell viability after PCA, 5-FU, and OXA treatment

The effect of PCA, 5-FU, and OXA alone on Caco-2 cell viability was shown in Fig. 1. According to the MTT test results, we observed no statistically significant decrease in cell viability after 24 h of PCA, 5-FU and OXA administration, and the IC_{50} dose could not be determined. PCA, 5-FU and OXA monotherapies were shown to reduce the viability of these cells in a concentration- and time-dependent manner. After 48-h exposure of PCA and 5-FU treatment, cell viability of ≤70% was obtained as of 50 µM dose, and the IC_{50} dose was determined as 694.1 µM and 982.8 µM, respectively. We observed the same cell viability from 10 µM dose of OXA treatment, where the IC_{50} dose was determined as 122.4 µM (Fig. 1). For combination doses of PCA, we administered 100 µM and 250 µM doses with 70% (P <0.05) viability over 48 h. In addition, ethyl alcohol, used as the solvent of PCA, was used at the highest concentration of 0.6%, where we observed 97.88% viability compared to that in the control group. For the combination doses of 5-FU, we administered 50 µM and 500 µM doses, which showed 68.1% (P <0.05) and 54.5% (P <0.05) viability compared to that in the control group in 48 h. For combination doses of OXA, we applied 10 µM and 50 µM doses, which showed 69.1% (P <0.05) and 53.6% (P <0.05) viability compared to that in the control group over 48 h.

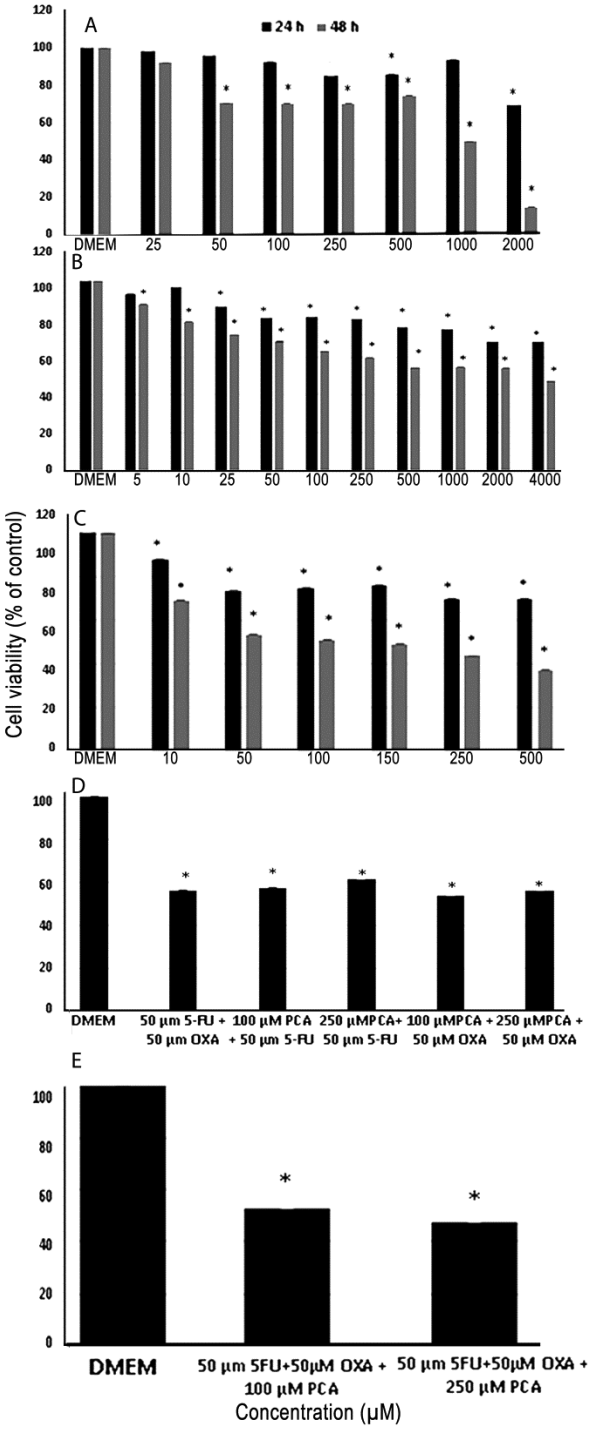

Fig. 1 — Cell viability tests of Caco-2 cells following (A) PCA; (B) 5-FU; and (C) OXA monotherapy. Effect of (D) double; and (E) triple combination therapy on Caco-2 cell viability (48 h) [*P <0.05 *vs*. control (n=6)]

Cell viability after PCA, 5-FU and OXA combination treatment

We observed statistically significant differences of cell viability for the dual combinations prepared with 100 µM and 250 µM PCA, 50 µM 5-FU and 50 µM OXA doses compared to that in the control group (P <0.05). Combinations containing 100 µM PCA showed significantly lower cell viability (54%) than did preparations 250 µM PCA alone (56%) P <0.05) While 50 µM 5-FU alone showed 75.4% viability, it was 57.3% in its dual combination applied with 100 µM PCA. While administration of 50 µM OXA showed 53.6% viability, its combination with 100 µM PCA showed 53.7% viability. We detected statistically significant differences of cell viability in triple combination groups compared to that in the control (P <0.05). While the viability was 56.3% in the double combination of 50 µM 5-FU and 50 µM OXA and 52.6% in 50 µM 5-FU + 50 µM OXA + 100 µM PCA, it was 47.1% with the combination of 50 µM 5-FU + 50 µM OXA + 250 µM PCA, with no statistically significant difference (P >0.05, Fig. 1).

Synergistic effect of combination therapy

The CI and DRI values obtained after 48 h of treatment are given in Table 1. The CI value was 0.44 when PCA+5-FU treatment was applied at a ratio of 2:1, while the CI value was 0.62 when PCA+OXA was applied at a ratio of 2:1. In the triple combination treatment, when 5FU+ OXA + PCA was applied at 1:1:2 and 1:1:5 ratios, the CI values were 0.67 and 0.69, respectively.

Table 1 — Combination index (CI) and dose reduction index (DRI) values for 5-Fu, OXA and PCA combinations in Caco-2 cell line

Treatment (48 h)	CI	DRI	Doses of individual drugs	Inter-pretation
5-FU+OXA	1,07	12,621	50 µm 5-FU	Nearly
		1,0	50 µm OXA	additive
PCA + 5-FU	0,44	2,52	100 µM PCA	Synergism
		21,59	50 µm 5-FU	
PCA+ 5-FU	1,28	0,89	250 µM PCA	Moderate
		5,71	50 µm 5-FU	antagonism
PCA + OXA	0,62	3,5	100 µM PCA	Synergism
		2,9	50 µM OXA	
PCA + OXA	1,55	1,48	250 µM PCA	Antagonism
		1,13	50 µM OXA	
5FU+ OXA + PCA	0,67	40,92	50 µm 5FU	Synergism
		2,87	50µM OXA	
		3,33	100 µM PCA	
5-FU+ OXA + PCA	0,69	50,17	50 µm 5-FU	Synergism
		3,03	50µM OXA	
		2,85	250 µM PCA	

[5-FU, 5-Fluorouracil; OXA, Oxaliplatin; and PCA, Protocatechuic acid]

Apoptosis

Mono- and oligo- nucleosome enrichment of cells incubated for 48 h with applied drug concentrations and DMEM used as a negative control was accepted as 1.00 for the DMEM (control cells) sample, and the enrichment factors of other samples were calculated proportionally. Compared to that in the control group, the enrichment factor in cells incubated for 48 h were 6.0 for 50 µM OXA, 4.0 for 100 µM PCA, 3.5 for 50 µM 5-FU + 50 µM OXA, and 4.6 for 50 µM 5-FU + 50 µM OXA + 100 µM PCA (P <0.05) (Fig. 2).

Gene expressions

MMP-2 and MMP-9 gene expression results are shown in Fig. 3. Administration of 50 µM 5-FU, 100 µM PCA and 250 µM PCA was associated with significantly lower expression of MMP-2 mRNA compared to that in the control group (0.2, 0.6, and 0.7-fold, respectively; P <0.05 for each pairwise comparison). 50 µM OXA + 100 µM PCA was associated with significantly higher expression of MMP-2 mRNA than that in the control group (1.6-fold, P <0.05). Compared to that in the control group, 5 µM OXA and 50 µM 5-FU + 50 µM OXA +100 µM PCA group had significantly higher expression of MMP-9 mRNA (2.1-fold and 3.0-fold,

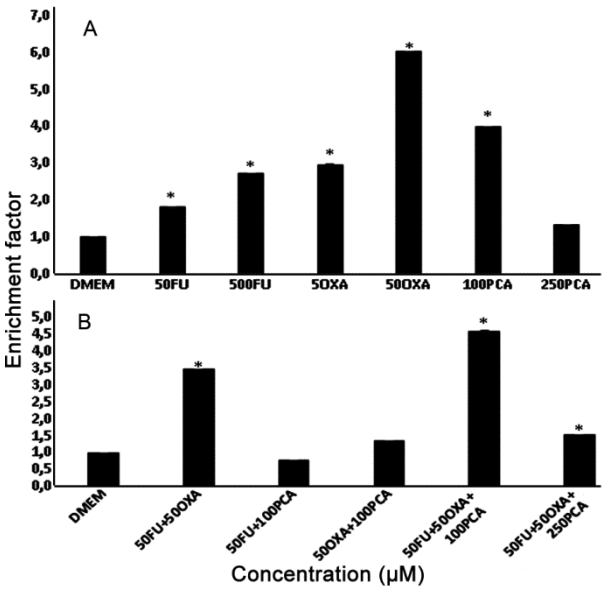

Fig. 2 — Apoptotic effect of PCA, 5-FU, and OXA alone and in combination on Caco-2 cells. [The X-axis of the graph represents the concentrations of the DMEM group and drugs, and the Y-axis represents the enrichment factor calculated from the ratio of the average absorbance values of each sample to the absorbance value of the control. *P <0.05 *vs*. DMEM. Data presented as mean ± standard deviation (n=3)]

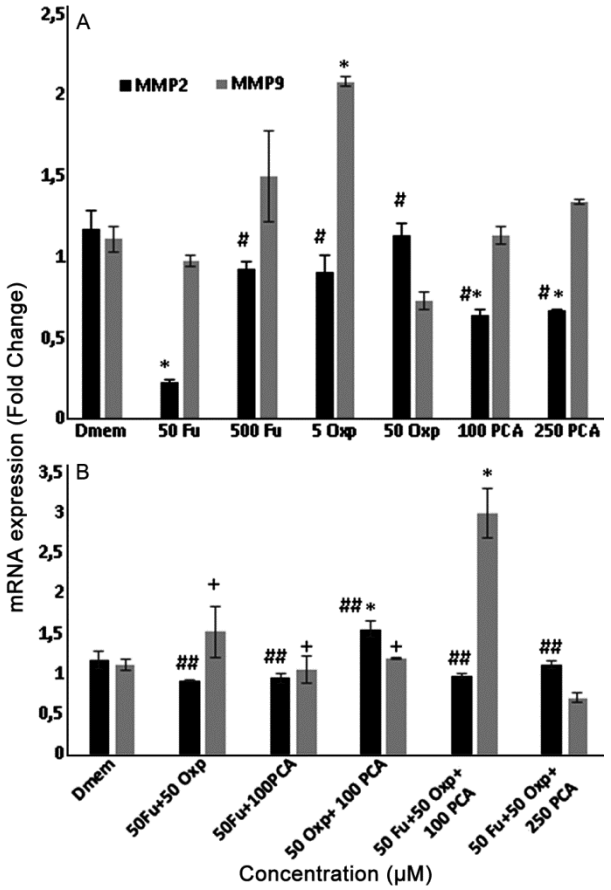

Fig. 3 — MMP-2 and MMP-9 mRNA expression levels of Caco-2 cells. [Mean value ± standard deviation (n=3) **P* <0.05 *vs*. DMEM, $^{\#}P$ <0.05 *vs*. 50 Fu, $^{\#\#}P$<0.05 *vs*. 50 Oxa+100 PCA, +*P* <0.05 *vs*. 50 Fu+50 Oxp+100 PCA]

respectively; *P* <0.05 for each pairwise comparison. Administration of 50 µM 5-FU +50 µM OXA +100 µM PCA was associated with 3.0-fold higher MMP-9 mRNA expression compared to that in all groups (*P* <0.05), (Fig. 3).

Discussion

Colon cancer is the third most common type of cancer worldwide with an increasing incidence every year. Different chemotherapy drugs and treatments are used in the treatment of the disease. However, the high burden of adverse effects of the drugs and/or the resistance of cancer cells to the drugs in time affect the treatment negatively[3,24].

One of the widely used combination with antitumor potential in the treatment of colon cancer is 5-FU/OXA[7]. However, resistance to 5-FU and OXA and toxicity at high doses attenuate their clinical efficacy. Therefore, there is a need for novel therapeutic options to reduce the drug concentration and side effects of 5-FU and OXA. Today, it is still being investigated whether many plant-derived substances found in nature have cancer-preventing or antiproliferative effects on cancer cells[25,26]. PCA is a phenolic compound commonly found in almost all dietary plants[27]. It has been reported in *in vitro* studies that PCA has antioxidant[28], antiatherosclerotic, anti-inflammatory, and anti-cancer activities, as well as cell proliferation suppressive effect on various cancer cell lines[12,29]. In our study, we tested the cytotoxic effect of 5-FU, OXA, PCA alone and their double and triple combinations on the human Caco-2 cell line and investigated the effects of the determined doses on MMP-2 and MMP-9 genes, which have an essential role in metastasis, on apoptosis. Our study seems to be the first to apply PCA in combination with 5-FU and OXA on Caco-2 cell line. In addition, the effect of PCA on human Caco-2 cells was demonstrated for the first time with this study.

According to MTT results, we determined that 100 µM PCA was more effective in combinations than 250 µM PCA dose. Our results showed that PCA causes mitochondrial damage in cells. Therefore, it was determined that the toxicity of PCA increased with increasing dose and time. Consistent with our findings, doses of PCA between 1-8 µmol/L were reported to exert similar cytotoxic effects on the human breast (MCF7), lung (A549), liver (HepG2), and cervical (HeLa) cancer cells, increasing with escalating doses. In the same study, the IC_{50} value of PCA on normal liver cells was found to be >30 mM[30]. Considering that this dose is relatively high, PCA seems to have a highly effective cytotoxic effect at higher doses on cancer cells. Moreover, PCA treatment was reported to reduce cell viability and colony formation of OVCAR-3, SKOV-3 and A2780 cells[31]. On the other hand, although the same cell line was used in some studies, the suppressive effect of PCA on cell proliferation varies considerably, which might be attributed to the difference in the length of the treatment period[29].

Combination therapy is based on the positive effects of interactions (synergistic or additive) between two or more drugs. In combination therapy, combined treatments based on compounds that exhibit synergistic or additive effects generally have less toxicity than monotherapy, as both compounds are given at lower doses[32,33]. Therefore, in our study, we investigated the synergistic effects of 5-Fu, OXA and PCA. Our results showed that different concentrations

of 5-FU, OXA and PCA inhibited the proliferation of Caco-2 cells in a dose-dependent manner. In addition, in the interaction analysis program, a synergistic effect was observed in Caco-2 cells with less than one CI and synergy in the combination treatment. Especially the synergistic effect between the combination of 5-FU and PCA was remarkable. This may be a suitable combined therapy to reduce the severe toxicity and side effects associated with 5-FU. Similar to our study, Motamedi *et al*.[34] showed that PCA interacts synergistically with other anticancer drugs in AGS (gastric adenocarcinoma) cells. On the other hand, many studies have shown that the combination of natural compounds with chemotherapeutic drugs enhance their antitumor activity through various mechanisms including induction of apoptosis, inhibition of cell proliferation, invasion and metastasis, which is consistent with the findings of our study[3,35,36].

Drugs considered to be used in cancer treatment are especially desired to cause apoptotic cell death. Apoptosis ensures that cancerous cells are destroyed before they enter the rapid proliferation process. However, cancer cells could escape apoptotic cell death via different mechanisms. Therefore, activating apoptotic cancer cell death is a promising target for cancer treatment. In colorimetric analysis, when 5-FU, OXA and PCA were applied alone, a slight increase in apoptosis was observed compared to the control group. Among all treatment groups, we observed the most significant increase of apoptosis in cells treated with 50 µM OXA and 5-FU + 50 µM OXA + 100 µM PCA. Apoptotic indices increased slightly when 5-FU, OXA, and PCA were administered alone compared to the control, while the increase was more pronounced when the drugs were administered in combination. In particular, we determined that 100 µM PCA increased the effectiveness of combined drugs.

Xie *et al*.[31] investigated the cytotoxic effect of PCA on the ovarian cancer cell line (OVAR-3), where PCA was reported to stop the cell cycle in the G2/M phase and regulate apoptosis with caspase-3 activation, Bax upregulation, and Bcl-2 downregulation. Similar studies in the literature reported comparable findings with our results[30]. We detected the presence of apoptotic activity via PCA on the Caco-2 cell line. Several studies have shown that PCA can induce apoptosis in cancer cells, which is consistent with the results of our study[34,37]. Considering the results, we can say that the administration of 5-FU, OXA and PCA causes dose-dependent suppression of cell proliferation and induces apoptosis. However, further studies are needed on the mechanisms by which PCA triggers apoptosis in Caco-2 cells.

The mechanisms of activation and regulation of MMP-2 and MMP-9 in cancer cells have not been fully elucidated yet. MMP2 and MMP-9 are localized to the tumor stroma in almost all cancer types, especially colon cancer[38]. We observed the highest increase in MMP-9 mRNA expression level in the group we applied a combination of 50 µM 5-FU + 50 µM OXA + 100 µM PCA. We detected a reduction in MMP-2 mRNA expression rates in all treatment groups compared to the control. Tsao *et al*.[39] reported declined MMP-2 and MMP-9 gene expression with PCA in various lung cancer cell lines. Another study reported a dose-dependent reduction of MMP2 and MMP9 expression with recombinant gensolin in colon cancer[40,41] consistent with our findings.

Conclusion

Our findings showed that the cytotoxic and apoptotic effects of PCA increased in a dose- and time-dependent manner at 48 hours. It also showed that PCA exerts a suppressive effect on Caco-2 cell proliferation based on mitochondrial activity, which might be mediated by activating apoptotic cell death. Moreover, the combination of 5-FU+OXA with PCA is not only a promising approach to potentially reduce the dose requirements of 5-FU+OXA but may also promote apoptosis. Our results suggest that PCA may be a useful agent in the prevention and/or treatment of colon cancer. However, the mechanism of action of PCA needs to be studied in more detail. Supported by further molecular-based studies, it can be determined through which pathways it acts in inducing apoptotic death in the cell.

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Conflict of interest

Authors declare no competing interests

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