Combined Effects of Protocatechuic Acid and 5-Fluorouracil on p53 Gene Expression and Apoptosis in Gastric Adenocarcinoma Cells

Öz

Amaç: Bu çalışma protokateşuik asit (PCA) ve 5-florourasilin (5-FU) gastrik adenokarsinoma (AGS) hücrelerine kombine etkisini değerlendirmiştir.

Gereç ve Yöntemler: 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolium bromid yöntemi, koloni oluşturma yöntemi, akış sitometrisi tekniği, gerçek zamanlı quantitatıve polymeraz zincir reaksiyonu ve Western blot yöntemleri 5-FU ve PCA’ya maruz kalan AGS hücrelerinde sitotoksisite, koloni oluşumu, apoptoz, p53 gene expression, ve Bcl-2 protein leveli AGS hücre proliferasyonu ve koloni oluşturunun inhibe ettiğini ve apoptozu artırdığını göstermiştir. Ek olarak, kullanılamayan kontrol hücrelerini p53 düzeyini artırmış ve Bcl-2 düzeyini azaltmıştır.

Sonuç: Sonuçlar göstermiştir ki AGS hücrelerinde kombine 5-FU/PCA maruziyeti koloni oluşumunu inhibe ederek antiproliferatif ve pro-apoptotik etkiler başlatabilir. Kombine 5-FU/PCA maruziyetinin etki gösterme mekanizması p53 geninin artması ve Bcl-2 geninin azalması ile ilişkilidir.

ABSTRACT

Objectives: This study evaluated the combined effects of protocatechuic acid (PCA) and 5-fluorouracil (5-FU) on gastric adenocarcinoma (AGS) cells.

Materials and Methods: The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, colony formation assay, flow cytometry technique, real-time quantitative polymerase chain reaction, and Western blotting were used to investigate cytotoxic effects, colony formation, apoptosis, p53 gene expression, and Bcl-2 protein level in AGS cells treated with 5-FU and PCA.

Results: Our results demonstrated that PCA (500 µM) alone or in combination with 5-FU (10 µM) inhibited AGS cell proliferation, inhibited a colony formation, and increased apoptosis compared with untreated control cells. Moreover, the combined 5-FU/PCA exposure led to upregulation of p53 and downregulation of Bcl-2 protein when compared to the untreated control cells.

Conclusion: The results demonstrate that the combined 5-FU/PCA may promote antiproliferative and pro-apoptotic effects with the inhibition of colony formation in AGS cells. The mechanisms by which the combined 5-FU/PCA exposure exerts its effects are associated with upregulation of p53 gene expression and downregulation of Bcl-2 level. Therefore, the combination of 5-FU with PCA not only could be a promising approach to potentially reduce the dose requirements of 5-FU but also could promote apoptosis via p53 and Bcl-2 signaling pathways.

Key words: Apoptosis, 5-fluorouracil, protocatechuic acid, gastric cancer, combination, colony formation
INTRODUCTION

Gastric cancer figures worldwide among the major issues faced by health systems due to its incidence and mortality rate. The 5-year survival rate for this disease is only about 20%. Gastric cancer’s prevalence varies in different geographic regions. Gastric cancer can be affected by predisposing factors to gastric carcinoma encompassing familial genetic background, smoking, inadequate intake of antioxidants, disproportionate salt intake, and infection by H. pylori. Furthermore, disorders such as gastritis, intestinal metaplasia, dysplasia, paraneoplastic lesions, and chronic atrophic gastritis are among the underlying causes of gastric cancer.

The therapeutic options for gastric carcinoma are primarily surgery, radiotherapy, and chemotherapy. 5-fluorouracil (5-FU) is a heterocyclic aromatic anticancer chemical agent that is widely used to handle various cancers by inhibiting the enzyme thymidylate synthase, preventing DNA replication. However, 5-FU has many side effects (diarrhea, stomatitis, emesis, neutropenia, inflammation of the mouth, loss of appetite, low blood cell counts, hair loss, and skin inflammation). Moreover, administration of 5-FU is frequently limited by dose-limiting toxicities. Interaction of chemotherapy with natural compounds may present a new perspective and an innovative strategy in cancer therapy. Interestingly, herbal compounds in tandem with 5-FU amplify the synergistic effects of administered therapeutics and exert cytotoxic effects specifically in tumor cells. Combined therapy with synergistic effects not only reduces the drug doses and resistance in chemotherapy but also decreases metastasis, raises the efficacy of 5-FU, and induces apoptosis. Apoptosis in cells is a type of programmed cell death under the control of factors such as p53 gene expression, which is mutated in most cancer cells. This gene plays a crucial role in genome stability, tumor suppression, induction of apoptosis, cell cycle stopping, and aging. In addition, p53 acts as a transcription factor for pre-apoptotic proteins.

Various experiments have been conducted to discover and use natural compounds for induction of apoptosis in cancer cells. Epidemiological studies have shown that a diet rich in phytochemical compounds is effective in inducing apoptosis in some cancers. Phytochemicals with antioxidant activity can inhibit carcinogenic processes in several models due to the expression of key proteins in signal transduction pathways and induction of apoptosis. It is also reported that many polyphenols can reduce the adverse effects of chemical therapies. Protocatechuic acid (PCA), also known as 3,4-dihydroxybenzoic acid, is a herbal phenolic acid mainly present in fruits, vegetables, and nuts and has anti-inflammatory, antibacterial, antihyperglycemic, anticancer, antinocer, and antispasmodic properties. Therefore, the aim of the present study was to assess the combined effects of 5-FU and PCA on p53 gene expression, colony formation, apoptosis, and Bcl-2 signaling protein level in the gastric adenocarcinoma (AGS) cell line.

MATERIALS AND METHODS

Chemicals and antibodies

The investigated human AGS cells were procured from the Pasteur Institute (Tehran, Iran). RPMI 1640 medium, trypsin 0.25%, penicillin/streptomycin (pen/strep), and fetal bovine serum (FBS) were supplied by Gibco (Rockville, MD, USA). Bcl-2 and β-actin primary antibodies were purchased from Elabscience Biotechnology Co. (Wuhan, China). PCA and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). 5-FU was purchased from Haupt Pharma (Wolftratshausen GmbH Co, Germany). The Roti®ZOL total RNA extraction kit was obtained from Carl Roth GmbH, Germany. The Annexin V-PI staining kit was purchased from BD Bioscience (California, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and p53 primers were purchased from Macrogen Company (South Korea). The other reagents used were of analytical grade.

Cell viability assay

AGS cells were cultured in 96-well plates (5000 cells per well) overnight in RPMI 1640 medium that contained 10% FBS and 1% pen/strep at 37°C in 98% humidity with 5% CO₂. Subsequently, the cells were treated with 5-FU (0-55 µM), PCA (0-1100 µM, solution in dimethyl sulfoxide (DMSO) with 0.1% final concentration), and the combination of 5-FU with PCA (10 µM and 500 µM, respectively) for 24 h. The medium was removed and the cells were incubated with MTT solution (5 mg/mL) for 4 h at 37°C. Afterwards, DMSO was added to each well of 96-well plates. The absorbance of each well was measured with a microplate reader (Stat Fax-2100, USA) at 490-570 nm. The percentage of cell viability was assessed as follows: viability= A (sample)/A (control)x100. At least three independent experiments were carried out.

Assessing the synergistic effects of 5-FU and PCA

The IC₅₀ values of 5-FU and PCA were used to determine synergistic effects between 5-FU and PCA through the combination index (CI) using the CI equation:

\[ CI = \frac{IC_{50_{5-FU}} + IC_{50_{PCA}}}{IC_{50_{5-FU}} + IC_{50_{PCA}}} \]

where A related to 5-FU concentration in combination with B, and B depicting PCA concentration in combination with A. Aₜ is the IC₅₀ of 5-FU and the Bₜ is the IC₅₀ of PCA. A CI value of 1 represents an additive effect, CI <1 indicates synergism, and CI >1 represents antagonism.
Colony formation assay
For the colony formation assay, AGS cells were cultured in 6-well plates (3x10^5 cells per well) overnight. The cells were then treated with 5-FU alone (10 µM), PCA alone (500 µM), and the combination of 5-FU and PCA (10 µM and 500 µM, respectively) and incubated at 37°C in an atmosphere of 5% CO_2 for 24 h. Then the medium was removed, while the cell culture medium was changed every 2 days for 14 days. Subsequently, the cells were washed with PBS and fixed with 70% ethanol and colonies were stained with 0.5% crystal violet. The number of colonies was counted and plating efficiency (PE) was calculated by the following formula: PE=(number of colonies/number of seeded cells) x100 and surviving fraction (SF) was determined by SF=(number of colonies/number of seeded cells x PE control) x100.

Apoptosis detection assay
The percentage of apoptosis and necrosis of cells were determined through flow cytometry using the AnnexinV-FITC Apoptosis Detection Kit (BD Bioscience, Franklin Lakes, NJ, USA). Briefly, AGS cells (2x10^5 per well) were cultured in 6-well plates and incubated overnight. The cells were treated with 5-FU (10 µM) and PCA (500 µM) or a combination of 5-FU and PCA (10 µM and 500 µM, respectively) for 24 h. Then the cells were harvested by trypsinization, washed with PBS, and stained with Annexin V for 20 min according to the manufacturer’s protocol at room temperature in the dark. The cells were analyzed using a FACSscan system (Becton-Dickinson and Company, San Jose, CA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)
RT-qPCR was then applied so as to assess the p53 gene expression in AGS cells. In summary, total RNA from each of the untreated control cells, 5-FU (10 µM), and PCA (500 µM) or the combination of 5-FU and PCA (10 µM and 500 µM, respectively) was extracted after 24 h of treatment using RoTi®ZOL solution according to the manufacturer’s instructions. The total mRNA concentration and quality of RNA were evaluated using OD measurements at 260/280 ratio using a Nanodrop 2000 spectrophotometer (Thermo-USA). For cDNA synthesis 1 µg of RNA was used with a synthesis kit (Takara Bio Inc., Japan) in the presence of specific primers. The sequences of the primers for the reaction were as follows: H-p53-F, forward 5'-CCCATCTCACAATCACCACAC-3' and reverse 5'-GACAAACACAGCCTCCTGAG3' and H-GAPDH-F, forward 5'ACACCACTCTCCTCAGCATC3', and reverse 5'GTCACCCCATCTGTTGCTGTA-3'. The primers were prepared with Oligo 6.0 software (Molecular Biology Insights, Cascade, CO, USA) and confirmed by BLAST (NCBI). The GAPDH gene was used as a reference gene for normalization. Enzyme activation was conducted for 10 min at 95°C, followed by 40 cycles of initial denaturation at 95°C for 10 s and annealing/extension at 62°C for 15 s and melting at 72°C for 20 s in a 3000 Rotor Gene (Corbett, Australia) real-time PCR system.

Western blotting
The AGS cells were grown in 6-cm dishes at the density of 6x10^5. After 24 h of treatment, protein extraction were performed for the control, 5-FU (10 µM), PCA (500 µM), and the combination of 5-FU and PCA (10 µM and 500 µM, respectively) using RIPA lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% Triton X-100, 1 mmol/L sodium orthovanadate, 0.5% sodium deoxycholate, 50 mmol/L NaF, 0.1% SDS, 1 mM EDTA, 0.1% sodium azide, 1 mmol/L phenylmethylsulfonyl fluoride, 50 µL of protease, and 250 µL of phosphatase inhibitor). The supernatants were collected and protein concentrations were determined using Bradford’s procedure. The Western blot procedure was described previously and primary antibodies Bcl-2 and β-actin were used according to the manufacturer’s protocols. β-actin was determined as an internal control. Then the blots were washed with TBS-Tween buffer 3 times for 10 min and they were incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 2 h and washed again as described above. Band intensity was evaluated using chemiluminescent reagents (ECL; Thermo Fisher Scientific, USA) and analyzed using ImageJ software.

Statistical analysis
The results of all experiments were expressed as mean ± standard deviation and the experiments were performed at least three times. SPSS (Version 20, SPSS Inc., Chicago, IL, USA) or GraphPad Prism 6 (Graphpad Software, San Diego, CA, USA) was used to perform the statistical analysis. Kruskal-Wallis analyses were used to assess between-group differences for the MTT assay, clonogenic assay, Annexin V assay, and RT-qPCR. For expression analysis, the relative levels of quantitative gene expression were calculated by the 2^-∆∆Ct method and the data were expressed as fold change. Melting curve analysis was performed after amplification to verify product identity. Western blotting was repeated 3 times. P values less than 0.05 were considered statistically significant for the differences between the groups. The CI was calculated using experimental CompuSyn software (CompuSyn Inc, Paramus, NJ, USA), and CI <1, =1, and >1 indicated synergism, additive effect, and antagonism, respectively.

RESULTS
Effects of 5-FU, PCA, and their combination on AGS cell viability
The result of the MTT assay demonstrated that 5-FU, PCA, and their combination can reduce the proliferation of AGS cells after 24 h (Figure 1). The IC_50 values of 5-FU and PCA alone were 40 µM and 700 µM, respectively (Figures 1A and 1B). The combination of 5-FU and PCA (10 µM and 500 µM, respectively) led to a synergistic CI equal to 0.6 with strong effects on AGS cell proliferation (Table 1). Moreover, the number of living cells decreased in the combination of 5-FU with PCA (10 µM and 500 µM, respectively) relative to untreated control cells and each agent alone (Figure 1C).
Clonogenic assay of AGS cells

The results of the colony formation assay demonstrated that the combination of 5-FU with PCA (10 µM and 500 µM, respectively) significantly decreased the colony numbers of AGS cells and the proliferation rate compared with those of the untreated control cells and 5-FU treated cells (Figure 2). After 14 days of cell culture, the number of colonies consisted of 63, 46, 30, and 22 for the control, 5-FU, PCA, and 5-FU/PCA combinations, respectively (Figure 2B). The SF for 5-FU, PCA, and 5-FU/PCA combination were 71%, 49%, and 34%, respectively. The results also showed PE in the control and treated experimental cells (Figure 2A).

Effects of 5-FU and PCA on apoptosis

The results of the flow cytometry showed the percentage of apoptosis and necrosis of 5-FU and PCA in AGS cells (Figure 3). Apoptosis in AGS cells was induced 17% by 10 µM 5-FU, 23% by 500 µM PCA, and 27% by the combination of 5-FU and PCA (10 µM and 500 µM, respectively). Apoptosis significantly increased (p<0.05) in the combination of 5-FU and PCA treatment when compared to that of the control and 5-FU treated cells (Figure 3).

Expression of p53 in AGS cells

The results of RT-qPCR showed p53 gene expression in the combination of 5-FU and PCA (10 µM and 500 µM, respectively). PCA and the 5-FU/PCA combination led to a significant increase (p<0.05) in p53 gene expression by almost 5.5- and 11.6- fold, respectively, in comparison with the control cells (Figure 4). No significant change was observed between 5-FU and untreated control cells.

Effects of 5-FU, PCA, and 5-FU/PCA combination on Bcl-2 signaling protein in AGS cells

The result of Western blotting demonstrated that the protein expression level of Bcl-2 markedly decreased after treatment with the 5-FU/PCA combination in AGS cells when compared to that of the untreated control cells and 5-FU treated cells (Figure 5).

DISCUSSION

The prevalence of cancer is increasing worldwide and the growing rate of mortality is quite alarming. Nowadays, 5-FU-based chemotherapy is a widespread procedure in the treatment of a wide range of cancers, including gastric, colorectal, and breast cancers, due to its effect in the inhibition of thymidylate synthase. Combination therapy not only
amplifies chemotherapy’s effects on tumor cells at lower concentrations but also it causes little toxicity to normal cells. In the present study, the combined treatment of 5-FU with PCA had stronger antiproliferation effects than either agent alone (Figure 1). Several previous studies have shown that PCA alone can decrease cell proliferation and viability in some cancer cell lines such as breast, lung, liver, cervix, and prostate cancer cells, which is in line with the findings in the present study. On the other hand, many studies have demonstrated that the combination of natural compounds with chemotherapeutic drugs enhanced their antitumor efficacy through various mechanisms, including cell sensitization, induction of apoptosis, inhibition of cell proliferation, invasion, metastasis, and angiogenesis, which is in agreement with the findings of our study. It has been reported that natural compounds can disperse vimentin, an epithelial-mesenchymal transition factor, and cause loss of cytoplasmic integrity. These compounds can make changes in cellular morphology through destabilization of the nucleus, cytoskeleton, mitotic spindle, and cell flexibility. Moreover, previous studies have shown that some antioxidants such as curcumin, resveratrol, and epigallocatechin-3-gallate not only have chemopreventive or chemotherapeutic effects but also they act as chemosensitizers on tumor cells. Therefore, in the present study it seems that PCA, at least partly, sensitized the AGS tumor cells to 5-FU, which led to increased antiproliferation and cytotoxic efficiency of 5-FU.

Figure 3. Induction of apoptosis after 24 h exposure to 5-FU (10 µM), PCA (500 µM), and combination of 5-FU and PCA (10 µM and 500 µM, respectively). Data were analyzed by FACScan and represent the mean of duplicate determinations. (A): Flow cytometry charts of Annexin V-FITC/PI staining in untreated (control) AGS cells and cells treated with 5-FU, PCA, and combination of 5-FU and PCA. (B): The percentage of apoptotic AGS cells. The results are expressed as mean ± SD of three separate experiments.

Figure 4. The gene expression of p53 in the presence or absence of 5-FU, PCA, and 5-FU/PCA combination on the AGS cells. Cells were exposed to a combination of 5-FU and PCA (10 µM and 500 µM, respectively). The expression of p53 was normalized with GAPDH as an internal standard. Our data demonstrated that the combined 5-FU and PCA resulted in a decrease in the number of colonies when compared to untreated control cells and each agent alone (Figure 2), which was in agreement with previous studies results. A previous study showed that combined 5-FU, cisplatin, and curcumin enhanced the anticancer effects of 5-FU in human
gastric cancer MGC-803 cells by decreasing cell viability, inhibiting colony formation, and inducing apoptosis, which is in line with the present study findings. It has also been reported that glabridin, the major isoflavane in licorice root, may inhibit the malignant proliferation of the human gastric cancer MKN-45 cell line and enhance the efficiency of 5-FU. The reduction in the number of colonies in the present study through combined 5-FU and PCA, at least partly, may have resulted from synergistic effects due to loss of sensitized cytoplasmic integrity and cellular changes such as Bcl-2 reduction, p53 elevation, and cell morphology.

Our flow cytometry results demonstrated that treatment with PCA and 5-FU combined resulted in increasing apoptosis in AGS cells compared to the untreated control cells (Figure 3). Antioxidants are used as anticancer compounds and can lead to cell death by activating the internal or external pathways of apoptosis. It has been demonstrated in several studies that PCA can induce apoptosis in cancer cells, which is in line with the results of the present study. In addition, PCA-induced apoptosis was found to be associated with the inhibition of Bcl-2, the mitochondrial translocation of Bax and Bid, and the cytosolic release of cytochrome C, which is in agreement with our study findings. On the other hand, it has been found that 5-FU induces apoptosis in cancer cells though p53 and troxerutin, a flavonoid, results in a dose-dependent suppression of cell proliferation and induces apoptosis, which is in line with our findings (Figures 1 and 3). In addition, it is reported that antioxidants can reduce the side effects and potential harmful impact of medications, influence multidrug resistance genes, which are responsible for resistance to different cytotoxic drugs, and enhance the residence time of chemotherapeutic drugs in cancer cells. Therefore, in the present study, the elevated efficacy of 5-FU for AGS apoptosis in the presence of PCA, at least partly, is due to PCA antioxidant capacity through sensitized cytoplasmic integrity and cellular changes such as Bcl-2 reduction and p53 elevation, and can trigger other internal or external signaling pathways of apoptosis.

p53 acts as a transcription factor for a series of pro-apoptotic proteins (such as Bad, Bax, and Bid) and anti-apoptotic Bcl-2 signaling protein and induces apoptosis by releasing cytochrome C. Natural antioxidants can cause cell death by controlling members of the Bcl-2 family and promoting DNA damage. In addition, it has been demonstrated that Bcl-2, which encodes an inner mitochondrial protein, can antagonize apoptosis in many tumor cells. Our results showed that the combined 5-FU/PCA increased p53 gene expression and decreased cellular Bcl-2 signaling protein (Figures 4 and 5). In many previous studies, it was found that PCA has the potential to induce apoptosis, increase p53 gene expression, and cause a decline in Bcl-2 protein, which is in agreement with our findings. Nevertheless, in the present study, it seems that the combination of PCA with 5-FU can strongly increase p53 gene expression (Figure 4). In another study, it was demonstrated that PCA acted as an apoptotic inducer of leukemia by decreasing the phosphorylation of retinoblastoma and decreasing the expression of Bcl-2, which is in line with the present study. On the other hand, researchers determined that Hibiscus polyphenol-rich extract containing PCA caused apoptosis in human gastric carcinoma cells via p53 phosphorylation and the p38 MAPK/FasL cascade pathway. In addition, it has been demonstrated that natural antioxidants such as forbesione, lupeol, luteolin, and myricetin can induce synergistic, apoptotic, and antiproliferative effects with 5-FU through the elevation of p53 gene expression and decreasing of the cellular Bcl-2 signaling protein in some cancer cells, which is in line with our findings. Therefore, in the present study, the elevation in p53 gene expression and the reduction in Bcl-2 protein level in the presence of PCA, at least partly, may have resulted from the potential of PCA in cell sensitization to 5-FU by activating intracellular signaling pathways.

In the present study, we did not investigate the effects of the combined 5-FU/PCA treatment on other cellular signaling pathways such as FAK, MAPK, MMP, COX, JNK, Akt, ERK, NF-κb, or caspases modulating factors, which influence invasion, metastasis, and apoptosis. We also did not study cell survival factors such as Bcl-xL or cFLIP. Thus, we suggest that prospective researchers investigate the above factors in combined 5-FU/PCA in future studies.

Figure 5. The Bcl-2 level of signaling pathway proteins in the AGS cell line. Cells were treated with 5-FU (10 µM), PCA (500 µM), and 5-FU/PCA combination (10 µM and 500 µM, respectively) for 24 h. (A): Density chart of Bcl-2/β-actin and (B): Western Blots bands

\*p<0.05 vs. control cells, \*p<0.05 vs. 5-FU treated cells

Bcl-2: Bcl-2 protein 2, AGS: Gastric adenocarcinoma, 5-FU: 5-fluorouracil, PCA: Protocatechuic acid

Protocatechuic acid (PCA) is demonstrated to decrease cell viability, inhibit colony formation, and induce apoptosis in AGS cells through synergistic effects due to loss of sensitized cytoplasmic integrity and cellular changes such as Bcl-2 reduction, p53 elevation, and cell morphology.
CONCLUSION

Our data indicate that the combined 5-FU/PCA treatment may promote antiproliferative and pro-apoptotic effects plus inhibition of colony formation in AGS cells. Some mechanisms by which the combined 5-FU/PCA treatment exerts its effects are associated with the upregulation of p53 and downregulation of Bcl-2 expression. Therefore, the combination of 5-FU with PCA not only could be a promising approach for potential reduction of dose requirements of 5-FU treatment but also could promote apoptosis via p53 and Bcl-2 signaling pathways.

ACKNOWLEDGEMENTS

We would like to express our gratitude to those who helped us in Clinical Biochemistry Research Center of Shahrekord University of Medical Sciences, Shahrekord, Iran. The results described in this paper were the MS dissertation of Ms. Zahra Motamedi. This study was funded by Shahrekord University of Medical Sciences, Shahrekord, Iran.

Conflicts of interest: No conflict of interest was declared by the authors. The authors alone are responsible for the content and writing of the paper.

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