Original Article

Asian Pacific Journal of Tropical Biomedicine

apjtb.org

doi: 10.4103/2221−1691.372286

Impact Factor® 1.51

Ponatinib and gossypol act in synergy to suppress colorectal cancer cells by modulating apoptosis/autophagy crosstalk and inhibiting the FGF19/FGFR4 axis

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ABSTRACT

Objective: To evaluate the efficacy of ponatinib plus gossypol against colorectal cancer HCT-116 and Caco-2 cells.

Methods: Cells were treated with ponatinib and/or gossypol at increasing concentrations to evaluate synergistic drug interactions by combination index. Cell viability, FGF19/FGFR4, and apoptotic and autophagic cell death were studied.

Results: Ponatinib (1.25-40 μM) and gossypol (2.5-80 μM) monotherapy inhibited HCT-116 and Caco-2 cell viability in a dose- and time-dependent manner. The combination of ponatinib and gossypol at a ratio of 1 to 2 significantly decreased cell viability ($P<0.05$), with a > 2- and > 4-fold reduction in IC_{50}, respectively, after 24 h and 48 h, as compared to the IC_{50} of ponatinib. Lower combined concentrations showed greater synergism (combination index<1) with a higher ponatinib dose reduction index. Moreover, ponatinib plus gossypol induced morphological changes in HCT-116 and Caco-2 cells, increased *beclin−1* and caspase-3, and decreased *FGF19*, *FGFR4*, *Bcl−2* and p-Akt as compared to treatment with drugs alone.

Conclusions: Gossypol enhances ponatinib's anticancer effects against colorectal cancer cells through antiproliferative, apoptotic, and autophagic mechanisms. This may open the way for the future use of ponatinib at lower doses with gossypol as a potentially safer targeted strategy for colorectal cancer treatment.

KEYWORDS: Autophagy; Apoptosis; Cell viability; FGF19/FGFR4; Gossypol; Ponatinib; HCT-116; Caco-2; Colorectal cancer

1. Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide and the second leading cause of cancer death, accounting for 1.8 million new cases and 861 000 deaths in 2020[1]. Fibroblast growth factor receptor 4 (FGFR4), a type of tyrosine kinase receptor, is strongly activated and involved in cell proliferation, migration, and differentiation in a variety of cancers, and this activation is intimately linked to its specific ligand, FGF19. So, the discovery and therapeutic evaluation of FGFR4-specific inhibitors is a hot topic[2]. Autophagy and apoptosis are two evolutionarily conserved programmed cell death mechanisms that are dysregulated in cancer cells, and they have a cross-talk relationship in antitumor therapy[3]. Chemotherapy is one of the most commonly used CRC treatments;

Significance

Combination therapy is a promising anticancer treatment strategy. This study found that a novel ponatinib and gossypol combination had a synergistic cytotoxic effect on colorectal cancer cells by inhibiting the FGF19/FGFR4 axis and modulating apoptosis-autophagy crosstalk. This was associated with a higher ponatinib dose reduction index, which may allow for the future use of lower and safer ponatinib dosages in the treatment of colorectal cancer cells. Further *in vivo* and clinical investigations are required for verifying its use.

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How to cite this article: El-Lakkany NM, Elkattan HH, Elsisi AE. Ponatinib and gossypol act in synergy to suppress colorectal cancer cells by modulating apoptosis/autophagy crosstalk and inhibiting the FGF19/FGFR4 axis. Asian Pac J Trop Biomed 2023; 13(3): 131-138.

Article history: Received 27 December 2022; Revision 1 February 2023; Accepted 3 March 2023; Available online 31 March 2023

nevertheless, it has some limitations, including established systemic toxicity, an unsatisfactory response rate, unpredictable innate and acquired resistance, and limited tumor-specific selectivity[4]. Developing new drugs or alternative strategies to refine or even substitute existing CRC chemotherapy is therefore highly desirable. Ponatinib, a potent tyrosine kinase inhibitor, is used as a third-line treatment for chronic myeloid leukemia. Because it inhibits FGFRs, AKT, ERK1/2, and other kinases, it has been tested in a variety of tumors where these kinases play important roles[5]. Given the association between ponatinib and vascular occlusive diseases and heart failure, it must be used in combination with other drugs to reduce the effective chemotherapeutic dose and hence its side effects while maintaining anticancer efficacy. Combination therapy is a promising strategy for synergistic anticancer treatment because it targets multiple cancer pathways simultaneously and employs distinct modes of action to prevent tumor drug resistance[6], one of the key challenges in CRC treatment. Gossypol is a polyphenolic compound found in cottonseed products that have antiviral, antibacterial, antioxidant, and anticancer activities[7]. Gossypol is thought to inhibit Bcl-2/Bcl-xL interaction with beclin-1 or Bax, and it can trigger autophagic and apoptotic cell death[8]. Our preliminary proof-of-concept investigation showed that the unique combination of ponatinib and gossypol had a synergistic anticancer impact against murine solid Ehrlich tumor[9]. It increased apoptotic markers such as p53, Bax, and caspase-9 while decreasing anti-apoptotic markers like Bcl-2. Furthermore, it significantly reduced the proliferative and angiogenic markers, FGFR4 and VEGF, respectively, as well as neoplastic cells, mitotic figures, and tumor giant cells, indicating the ability to suppress cancer growth/persistence. In the current study, we intended to continue using this new drug combination to do more detailed pharmacologic, drug interaction, and mechanistic research on colorectal cancer HCT-116 and Caco-2 cell lines.

2. Materials and methods

2.1. Drugs

Ponatinib (943319-70-8) and gossypol-acetic acid (12542-36-8) were purchased from BOC Science, BOCSCI Inc, 45-16 Ramsey Road Shirley New York, NY 11967, USA, and used in this study.

2.2. Cell lines and culture

The human CRC cell lines (HCT-116 and Caco-2; VACSERA, Dokki, Giza, Egypt) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Life Technologies, America) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco), and kept in a humidified incubator (Vision Scientific Co., Ltd., Korea) with 95% air and 5% CO_2 at 37 ℃. The media were continuously changed every 3-4 d, and the cells passaged after reaching 80% confluence.

2.3. Cell viability assay

The cytotoxic effect of ponatinib, gossypol, and their combinations on CRC cell lines was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, HCT-116 and Caco-2 cells (1×10^5 cells/well) were seeded in 96-well tissue culture plates (100 µL/well) and incubated at 37 ℃ for 24 h to develop a complete monolayer sheet (80% confluency). Then, it was replaced with a fresh medium containing ponatinib (1.25-40 µM), gossypol (2.5-80 µM), and in combinations (at a constant ratio of 1 to 2) dissolved in vehicle dimethyl sulfoxide (0.1%), each concentration was done in triplicates. The plates were incubated for 24 h and 48 h at 37 ℃, and the media were then aspirated; the cells were rinsed with phosphate buffered saline (PBS), and 20 µL of the MTT solution (5 mg/mL in PBS, Bio Basic Canada INC.), and incubated for another 2-4 h at 37 ℃ in 5% CO_2. The experiment was independently performed 3 times. The optical density (OD) of the MTT formazan was measured at 570 nm using a microplate reader (Mindray MR-96A). The cell viability percentage was calculated according to the equation: (OD of treated cells)/(OD of untreated cells)×100. The IC_{50} (dose of ponatinib and gossypol required to inhibit cell growth by 50%) was assessed using nonlinear regression analysis (GraphPad Software Instat, version 5; Inc., La Jolla, CA, USA).

2.4. Drug combination and synergy analysis

The drug-drug interaction between ponatinib and gossypol was evaluated using the computer software CompuSyn (version 1.0.1) to calculate the fraction of cells affected (fa), combination index (CI), and dose reduction index (DRI) for the drugs in combination. The CI was calculated using the median-effect principle and the isobologram technique, as modified from the multiple-drug effect analysis[10], with CI < 1 denoting synergism, CI = 1 designating additive effect, and CI > 1 indicating antagonism. DRI denotes how many folds of dose reduction are allowed for each drug due to synergism when compared with the dose of each drug alone where DRI > 1 indicates a greater dose reduction for a given therapeutic effect.

After that, HCT-116 and Caco-2 cells were cultured in 24-well plates at a density of 5×10^4 cells/well and treated with ponatinib (IC_{50}), gossypol (IC_{50}), or a combination of both for 48 h. These cells were examined under an inverted microscope for any morphological changes characteristic of cell toxicity. Morphological images of the cells were captured using phase contrast microscopy (Reichert jung, Nikon Eclipse TS200, Nikon) at a magnification of ×10, and compared with those of the untreated control cells. The cells were then harvested and resuspended in either 500 µL triazole for PCR

analysis (*FGF19*/*FGFR4*, *beclin–1*, and *Bcl–2*) or PBS (pH. 7.4) for ELISA assays (caspase-3 and p-Akt).

2.5. Assessment of beclin–1, FGF19, FGFR4 and Bcl–2 gene expressions via RT–PCR

Cell pellets were treated with Qiazol reagent (Qiagen, Germany), and total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration and purity of total RNA were determined using a nanodrop and measuring absorbance at 260 and 280 nm (1.8-2.0). First-strand complementary DNA (cDNA) was generated, and RT-PCR amplification and analysis were carried out in an optical 96-well plate (light cycler 480 Ⅱ) according to the manufacturer's instructions using the HERA SYBR® Green RT-qPCR Kit (WF1030300X). Table 1 shows the primers used for *beclin–1*[11], *FGF19*, *FGFR4*[12], *Bcl–2* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, an endogenous reference gene)[13]. The relative expression of selected genes was determined using the $2^{-\Delta\Delta Ct}$ method[14].

2.6. Assessment of p–Akt level and caspase–3 activity via ELISA

Cell pellet lysates were used for analysis of p-Akt (Catalogue No. 201-12-9003, Sun Red, China) and caspase-3 (Catalogue No. 201-12-0970, Sun Red, China) *via* ELISA technique in accordance with the manufacturer's instructions. First, the marker antigen was bound to the monoclonal antibody. After washing, an antigen-specific antibody worked as a detector by attaching it to the captured marker. Finally, a horseradish peroxidase (HRP) antirabbit IgG solution was added, followed by a substrate solution to produce the color.

2.7. Statistical analysis

Data are presented as mean ± SEM. To determine the significant difference between the mean values of different groups, a one-way ANOVA test followed by a Tukey *post–hoc* test (SPSS, software package version 16.0, Chicago, IL, USA) was used. P values < 0.05 were considered statistically significant.

Table 1. Primer sequences for real-time PCR analysis.

Genes	Primer sequence
FGF19	Forward primer: 5′-GCACAGTTTGCTGGAGATCA-3′
	Reverse primer: 5′-ATCTCCTCCTCGAAA GCACA-3′
FGFR4	Forward primer: 5′-AGCACCCTACTGGACACACC-3′
	Reverse primer: 5′-ACGCTCTCCATCACGAGACT-3′
Beclin–1	Forward primer: 5′-ACAGAGCTCATGGAAGGGTCTAAGACGTCC-3′
	Reverse primer: 5′-TACGAATTCTCATTTGTTATAAAATTGTG-3′
Bcl–2	Forward primer: 5′-ACTGGCTCTGTCTGAGTAAG-3′
	Reverse primer: 5′-CCTGATGCTCTGGGTAAC-3′
GAPDH	Forward primer: 5′-GGGAAGGTGAAGGTCGGAGT-3′
	Reverse primer: 5′-GGGGTCATTGATGGCAACA-3′

3. Results

3.1. Ponatinib and gossypol inhibits HCT–116 and Caco–2 cell viability

Both drugs inhibited the viability of HCT-116 and Caco-2 cells (P<0.05) in a dose- and time-dependent manner (Figure 1). Ponatinib and gossypol showed varying IC_{50} values against HCT-116 cells (4.65 μM and 12.09 μM, respectively at 24 h; 2.91 μM and 6.48 μM, respectively at 48 h). Similarly, adding ponatinib and gossypol to Caco-2 cells caused IC_{50} values of 16.57 μM and 26.90 μM, respectively after 24 h, and 9.87 μM and 18.95 μM, respectively, after 48 h. Of note, HCT-116 cells were found to be more sensitive to these drugs than Caco-2 cells.

The combination of ponatinib (1.25-40 μM) and gossypol (2.5-80 μM) at a constant ratio of 1 to 2 significantly decreased the viability of HCT-116 cells from 72% to 2% and 47% to 1.5% at 24 h and 48 h, respectively (P<0.05) (Figure 2), with a > 2-fold reduction in IC_{50} (2.24 μM & 1.06 μM) as compared to the IC_{50} of ponatinib-treated cells (4.65 μM & 2.91 μM, respectively). Similarly, the viability of Caco-2 cells was substantially decreased from 77% to 4% and 64% to 1.5% at 24 h and 48 h, respectively (P<0.05) (Figure 2), with a > 4-fold reduction in IC_{50} (4.18 & 2.01 μM) as compared to the IC_{50} of ponatinib-treated cells (16.57 μM & 9.87 μM, respectively).

3.2. Synergistic effects of ponatinib and gossypol in HCT–116 and Caco–2 cells

The effect of ponatinib plus gossypol on cell growth was then investigated to identify the pattern of interaction between the two drugs. Table 2 shows that the CI values in HCT-116 ranged from 0.72 to 1.07 after 24 h and from 0.56 to 0.87 after 48 h, and in Caco-2 cells from 0.39 to 0.63 after 24 h and from 0.21 to 0.47 after 48 h, indicating superior synergism at 48 h rather than 24 h, with DRI values for ponatinib being always > 1 at any combination point of two drugs. Furthermore, the combination of 2.5 μM ponatinib and 5 μM gossypol which represented approximately their IC_{50} values in HCT-116, resulted in 78% growth inhibition with CI = 0.56, demonstrating the greatest synergism with the higher DRI of ponatinib by 3.24 fold, whereas the combination of 10 μM ponatinib and 20 μM gossypol, which also constituted nearly their IC_{50} in Caco-2, resulted in 90% growth inhibition with stronger synergism (CI = 0.29) and 8.73 fold DRI of ponatinib (Table 2). Based on these findings, the combination of ponatinib (2.5 μM and 10 μM) and gossypol (5 μM and 20 μM) at IC_{50} concentrations in HCT-116 and Caco-2 cells was investigated at 48 h for morphological changes, FGF19/FGFR4 axis, as well as apoptotic and autophagic biomarkers.

Table 2. Combination index values of adding ponatinib to gossypol in HCT-116 and Caco-2 cells for 24 and 48 h.

Cell lines	PON (μM)	GOS (μM)	Fa 24 h	Fa 48 h	CI 24 h	CI 48 h	DRI PON 24 h	DRI PON 48 h
HCT-116	40	80	0.98	0.99	0.83	0.74	3.60	2.32
	20	40	0.93	0.96	1.04	0.80	2.11	2.17
	10	20	0.85	0.92	0.98	0.82	1.90	2.16
	5	10	0.74	0.82	0.84	0.87	1.99	2.07
	2.5	5	0.59	0.78	0.72	0.56	2.10	3.24
	1.25	2.5	0.28	0.53	1.07	0.74	1.23	2.49
Caco-2	40	80	0.96	0.98	0.39	0.21	10.89	14.38
	20	40	0.86	0.96	0.60	0.27	5.37	9.99
	10	20	0.74	0.90	0.57	0.29	4.87	8.73
	5	10	0.53	0.79	0.63	0.34	3.77	6.92
	2.5	5	0.34	0.55	0.62	0.47	3.37	4.61
	1.25	2.5	0.23	0.36	0.50	0.47	3.85	4.43

HCT-116 and Caco-2 cells were treated with the combination of ponatinib and gossypol at the doses indicated for 24 and 48 h. CI < 1, CI = 1, and CI > 1 indicate synergism, additivity, and antagonism, respectively. CI=(dA/DA) + (dB/DB), where dA and dB are the concentrations of PON and GOS in combination, whereas, DA and DB are the concentrations of PON or GOS, respectively, which produce the same effect alone. The computer software CompuSyn (version 1.0.1) was used for the determination of CI, DRI, and Fa for the drugs in combination. PON: ponatinib, GOS: gossypol, CI: combination index, DRI PON: dose reduction index of ponatinib, Fa: fraction affected.

3.3. Effect of ponatinib and/or gossypol on FGF19/FGFR4 axis

When HCT-116 and Caco-2 cells were treated separately with ponatinib (2.5 and 10 μM) and gossypol (5 and 20 μM) for 48 h, there was a significant (P<0.05) reduction in *FGF19* (68% & 42% in HCT-116 and 82% & 67% in Caco-2, respectively) and *FGFR4* (85% & 53% in HCT-116 and 91% & 74% in Caco-2, respectively) gene expression when compared to the control. Furthermore, ponatinib (2.5 and 10 μM) and gossypol (5 and 20 μM) combination caused a significant (P<0.05) reduction in *FGF19* and *FGFR4* gene expression by 96% and 98% in HCT-116 cells (Figure 3A and B) and 98% and 99% in Caco-2 cells (Figure 3C and D), respectively. Meanwhile, ponatinib (2.5 and 10 μM) and gossypol (5 and 20 μM) combination resulted in a greater synergistic reduction (P<0.05) in both *FGF19* and *FGFR4* gene expression than treatment with drugs alone (Figure 3A-D).

3.4. Effect of ponatinib and/or gossypol on apoptotic biomarkers (Bcl-2 and caspase-3)

Treatment with ponatinib (2.5 and 10 μM) and gossypol (5 and 20 μM) alone significantly reduced (P<0.05) *Bcl-2* gene expression by 58% and 68% in HCT-116 (Figure 4A) and 68% and 88% in Caco-2 (Figure 4B), respectively, while increasing caspase-3 protein expression by 1.59 and 1.82 fold in HCT-116 cells and 1.80 and 1.96 fold in Caco-2 cells, respectively, when compared to the control group. Moreover, ponatinib (2.5 and 10 μM) and gossypol (5 and 20 μM) combination caused a significant reduction (P<0.05) in *Bcl-2* gene expression and an increase in caspase-3 protein expression by

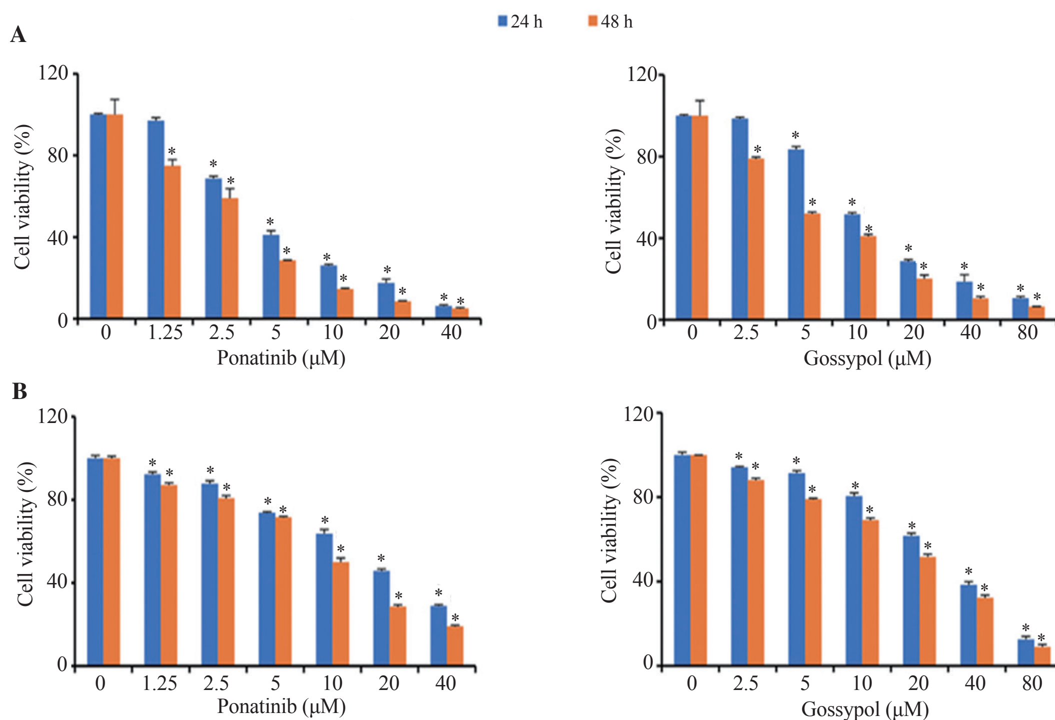

Figure 1. Effect of ponatinib and gossypol as single agent on the viability of (A) HCT-116 and (B) Caco-2 cells after 24 and 48 h. Data are presented as mean ± SEM and analyzed by a one-way ANOVA test followed by a Tukey *post-hoc* test. *Significantly different from the untreated control at P<0.05.

94% and 2.23 fold in HCT-116 cells and 99% and 2.50 fold in Caco-2 cells, respectively. Meanwhile, ponatinib plus gossypol demonstrated the most significant effect on reducing *Bcl−2* gene expression and increasing caspase-3 protein expression than treatment with drugs alone. These findings were linked to morphological changes observed in HCT-116 and Caco-2 cells (Supplementary Figure), with untreated cells forming a monolayer sheet with 80% confluence and a typical polygonal and intact appearance under a light microscope. However, cells co-treated with ponatinib (IC_{50})/gossypol (IC_{50}) appeared spare and detached completely from the plate surface, with progressive cytoplasmic shrinkage, granulation, and condensation (the typical morphologic signs of apoptosis).

3.5. Effect of ponatinib and/or gossypol on autophagic biomarkers (p−Akt and beclin−1)

Treatment with ponatinib (2.5 and 10 µM) and gossypol (5 and 20 µM) alone induced a significant reduction in p-Akt protein level by 34% and 29% in HCT-116 (Figure 4A) and 44% and 34% in Caco-2 (P<0.05) (Figure 4B), respectively, as well as an increase in *beclin−1* gene expression by 14.55 and 10.53 fold in HCT-116 and 17.39 and 12.55 fold in Caco-2, respectively when compared to the control. Moreover, ponatinib (2.5 and 10 µM) and gossypol (5 and 20 µM) combination significantly lowered p-Akt protein levels, while increasing *beclin−1* gene expression by 54% and 35.52 fold in HCT-

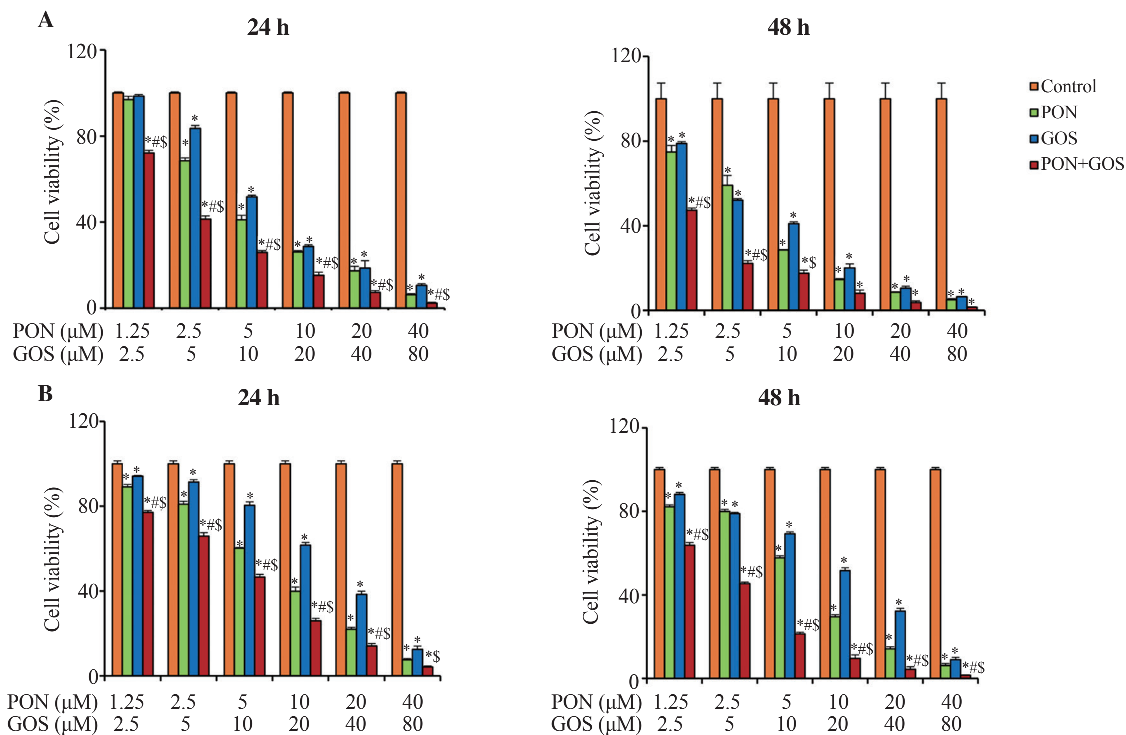

Figure 2. Effect of ponatinib (1.25-40 µM) plus gossypol (2.5-80 µM) at a ratio of 1:2 on the viability of (A) HCT-116 and (B) Caco-2 cells after 24 and 48 h. Data are presented as mean ± SEM and analyzed by a one-way ANOVA test followed by a Tukey *post−hoc* test. PON: ponatinib, GOS: gossypol. *, #, $Significantly different from the untreated control, PON, and GOS groups, respectively, at P<0.05.

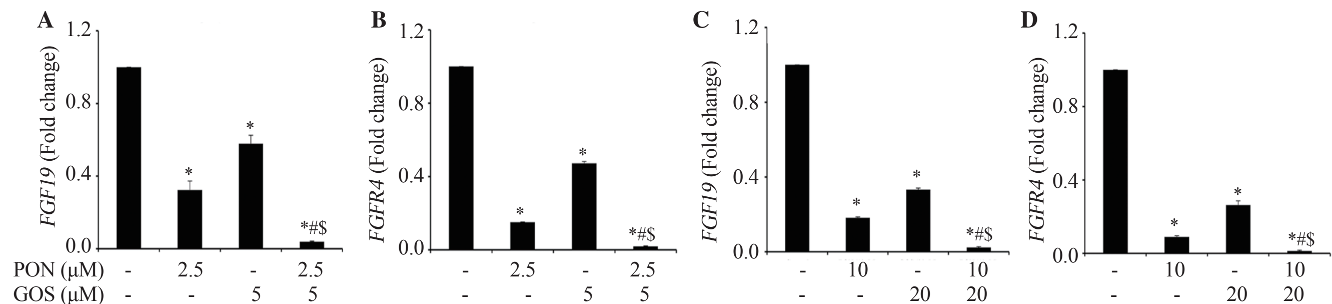

Figure 3. Effect of ponatinib and gossypol as single agent or in combination on the FGF19/FGFR4 axis after 48 h in HCT-116 (A, B) and Caco-2 (C, D) cells. Data are presented as mean ± SEM (n=3) and analyzed by a one-way ANOVA test followed by a Tukey *post−hoc* test. *, #, $Significantly different from the untreated control, PON, and GOS groups, respectively, at P<0.05.

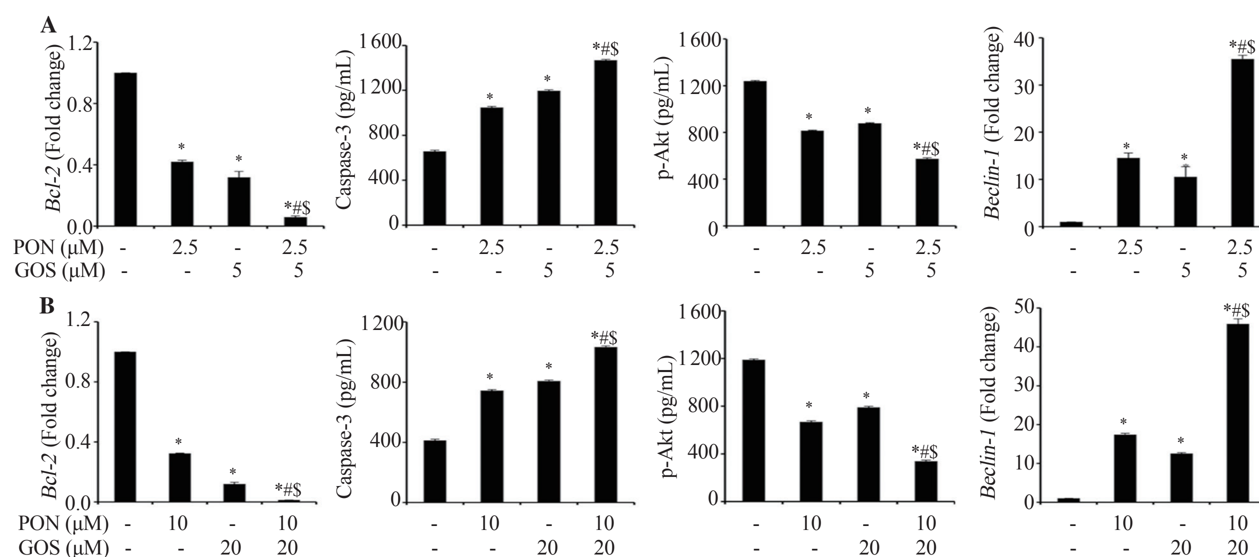

Figure 4. Effect of ponatinib and gossypol as single agent or in combination on apoptotic markers *Bcl–2* and caspase-3, as well as autophagic markers p-Akt and *beclin–1* in (A) HCT-116 and (B) Caco-2 cells. Data are presented as mean ± SEM (n=3) and analyzed by a one-way ANOVA test followed by a Tukey *post–hoc* test. *, #, $Significantly different from the untreated control, PON, and GOS groups, respectively, at P<0.05.

116 and 72% and 45.92 fold in Caco-2 cells, respectively (P<0.05), compared with treatment with drugs alone.

4. Discussion

In this study, we planned to use a novel ponatinib and gossypol combination to conduct pharmacologic, drug interaction, and mechanistic studies on colorectal cancer HCT-116 and Caco-2 cells. The FGF19/FGFR4 axis and autophagic and apoptotic biomarkers were explored to better understand the molecular mechanisms behind the potential cytotoxic effects of this combination. In this study, ponatinib and gossypol suppressed HCT-116 and Caco-2 cell proliferation in a dose- and time-dependent manner; however, co-treatment of both drugs at a constant ratio of 1 to 2 demonstrated a greater and synergistic inhibition of cell proliferation, as evidenced by a > 2- and > 4-fold reduction in IC_{50} values after 24 h and 48 h, respectively, as compared to IC_{50} of ponatinib-treated cells. Notably, the CI values were < 1 for the majority of the concentrations tested, indicating a synergistic effect. The combination of ponatinib and gossypol had a more significant effect on the inhibition of cell proliferation, particularly at low doses, which can avoid ponatinib toxicity to a greater extent. Importantly, ponatinib (2.5 μM and 10 μM) and gossypol (5 μM and 20 μM) combination at IC_{50} exhibited a strong synergism against HCT-116 and Caco-2 cells at 48 h, with superior ponatinib DRI values of 3.24 and 8.73, respectively. As a result, these IC_{50} values were chosen to investigate the biomarkers behind this synergistic antitumor effect.

Active FGF19-FGFR4 signaling is an oncogenic pathway in certain cancers including colorectal cancer[2]. FGFR4, a transcription factor, is required for embryonic development, tissue repair, tumor angiogenesis, cancer progression, and metastasis. FGF19 binds to FGFR4 selectively, and abnormal FGF19 signaling is translocated into tumor cells *via* a variety of oncogenic routes, resulting in tumor-promoting activity, with FGF19-FGFR4 binding activating downstream signaling cascades such as PI3K/AKT, apoptosis, and autophagy[15]. Thus, the FGF19-FGFR4 axis may be an intriguing target for effective anticancer therapies in CRC. Herein, ponatinib and gossypol combination at IC_{50} concentrations led to a synergistic suppression of FGF19/FGFR4 axis, resulting in a decrease in cell viability for both HCT-116 and Caco-2 cells. This result is consistent with the findings of Gao *et al.*[16], who found that ponatinib was effective on HCC cell lines by targeting the FGF19/FGFR4 axis. Similarly, both enantiomeric forms of gossypol inhibited the expression of growth factors with heparin-binding activity (bFGF-2 and FGFR-3)[17] and FGFR4[9]. FGFR4 knockdown reduces CRC cell migratory and invasive ability by upregulating epithelial marker E-cadherin and downregulating mesenchymal marker Snail, implying a pivotal role of FGFR4 in CRC metastasis[18].

Cell apoptosis is one of the major mechanisms of cell death in response to cancer therapy, and it is often produced *via* extrinsic and intrinsic pathways[19]. In this study, the combination of ponatinib and gossypol decreased Bcl-2, an antiapoptotic protein, and increased active caspase-3, a major protein regulator of apoptosis, compared to each drug alone, indicating enhanced apoptosis. This resulted in morphological changes in HCT-116 and Caco-2 cells. Hu *et al.* identified membrane alterations (*e.g.*, loss of membrane integrity), cytoplasmic content changes (*e.g.*, mitochondrial damage

and increased cytoplasmic shrinkage), and nuclear condensation as hallmarks of apoptosis[20]. Bcl-2 inhibits cell apoptosis by decreasing mitochondrial cytochrome c release, thereby preventing caspase-3 activation[21]. In response to pro-apoptotic signals, activation of effector caspases such as caspase-3 needs activation of initiator caspases such as caspase-9[22]. Caspase-3 changes in response to ponatinib plus gossypol treatment may thus be associated with caspase-9 changes, which is consistent with the findings of El-Lakkany *et al.*[9], who found that this unique combination had a synergistic apoptotic impact in solid Ehrlich carcinoma by targeting Bcl2/Bax/caspase-9 *via* the mitochondrial pathway. Gossypol increases caspase-3 while decreasing Bcl-2 in AtT20 cells, promoting apoptosis[23]. Likewise, ponatinib inhibited Bcl-2 in SK-Hep-1 and SNU-423 cells in a concentration-dependent manner[24]. FGFR4 depletion in this study was associated with decreased Bcl-2, increased caspase-3, and enhanced apoptosis. Of note, FGFR depletion was found to be FLIP- and Bax-dependent, resulting in caspase 8 activation, which then cross-talks with the mitochondria to promote Bax-mediated cytochrome c release and caspase 9 activation, followed by executioner caspases 3 and 7, resulting in apoptosis[25]. Taken together, we assume that the inhibitory action of ponatinib and gossypol on FGFR may play a role in their apoptotic effects, but the likely mechanism requires further investigation.

Aside from apoptosis, autophagy is one of the proposed cell death pathways in cancer therapy, and it can either direct or collaborate with apoptosis to generate cell death. P-Akt and beclin-1 are two of the most well-known autophagic indicators. The phosphorylation of beclin-1 by Akt plays a role in autophagy suppression and oncogenesis[26]. Treatment with ponatinib and gossypol markedly lowered p-Akt protein levels and elevated *beclin-1* expression. Beclin-1, an important autophagy-inducing protein, is linked to and inhibited by Bcl-2 or its homolog Bcl-xL, suggesting that the interaction between the core mechanisms regulating apoptosis and autophagy may focus on beclin-1 or Bcl-2[27]. Overall, we believe that the interaction between beclin-1 and Bcl-2 in this study was crucial in regulating the crosstalk between autophagy and apoptosis, but the likely mechanism requires additional exploration. Previously, it was discovered that decreasing Bcl-2 makes beclin-1 more accessible, thereby increasing beclin-1-dependent autophagy[28]. Gossypol is a BH3 mimetic compound that binds to the BH3 groove of anti-apoptotic Bcl-2 proteins, preventing Bcl-2/Bcl-xL from interacting with beclin-1 or Bax, and regulating apoptosis and autophagy[29]. Furthermore, gossypol was shown to inhibit Bcl-2 and beclin-1 interaction at the endoplasmic reticulum while increasing beclin-1 expression in cancer cells *via* the beclin-1 Atg5-dependent autophagic pathway[30].

According to the findings of this study, a novel combination of ponatinib and gossypol is a potential therapeutic strategy against HCT-116 and Caco-2 cells by synergistically blocking the FGF19/FGFR4 axis and inducing apoptosis and autophagy. This synergistic effect was associated with increased ponatinib DRI, which may allow for the use of lower and safer ponatinib dosages, despite the fact that it has been linked to vascular occlusive diseases and heart failure. However, this study has some limitations. The synergistic effect of ponatinib plus gossypol was only tested after 48 h. So, this effect must be investigated in a long-term colony formation assay (at least 14 d) using lower concentrations of these drugs. Besides, more in-depth parameters are required to investigate the autophagy-apoptosis crosstalk underlying this synergistic effect. In addition, we must examine the effects of ponatinib, gossypol, or a combination of the two drugs on human healthy epithelial colon cells to determine their off-target effects. Overall, this novel combination may offer a promising adjuvant targeted chemotherapy in the treatment of CRC.

Conflict of interest statement

The authors declare no conflict of interest.

Funding

This study received partial financial support from the Theodor Bilharz Research Institute, Warrak El-Hadar, Imbaba, Giza 12411, Egypt.

Authors' contributions

NE contributed to the study's conceptualization and design, data analysis and interpretation, drafting, and critical revision of the manuscript. HE contributed to the study's conceptualization, carried out the experiments, collected data, and wrote the first draft of the manuscript. AE supervised the study and contributed to the final version of the manuscript. All authors read and approved the manuscript.

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