**Original Article**

**Synergistic antitumor effect of 5-fluorouracil and withaferin-A induces endoplasmic reticulum stress-mediated autophagy and apoptosis in colorectal cancer cells**

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**Abstract:** The development of chemo-resistance against 5-fluorouracil (5-FU) in tumor cells is one of the main debacles in colorectal cancer (CRC) patients. A recent combination of 5-FU with oxaliplatin or cetuximab drastically improves the survival rate in CRC patients; however, the toxicity issue cannot be evaded completely. Thus, searching for novel drug combinations with high specificity and low toxicity is seemingly important. Owing to the less undesirable effects of natural products on normal cells, here we investigated the synergistic antitumor effect of withaferin-A (WA) in combination with 5-FU. Our results demonstrate that the combination of WA and 5-FU induces a significant antiproliferative effect and modulates endoplasmic reticulum (ER) stress in favor of cell death in colorectal cancer (CRC) cells. Mechanistically, the combination upregulates the expression of ER stress sensors (BiP, PERK, CHOP, ATF-4, and eIF2α) and executes PERK axis mediated apoptosis in CRC cells. Additionally, the combined treatment of WA and 5-FU mediated ER stress induces autophagy and apoptosis, which were confirmed by immunoblotting, acridine orange (AO) staining and annexin-V FITC by flow cytometry. In contrast, inhibition of ER stress with salubrinal significantly decreases both autophagic and apoptotic cell populations. Moreover, pharmacological inhibition of either autophagy or apoptosis by their respective inhibitors 3-methyladenine (3-MA) or carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoro-methyl ketone (Z-VAD-FMK) decreases their respective population of cells but could not affect either of the population significantly. Finally, the combination attenuates the expression of β-catenin pathway associated proteins and arrests cell cycle at the G2M phase in CRC cells. In summary, the combination of WA and 5-FU decreases cell viability by inducing ER stress-mediated induction of autophagy and apoptosis, inhibiting the β-catenin pathway and arresting the cell cycle at a G2M phase in CRC cells.

**Keywords:** Synergistic effect, endoplasmic reticulum, autophagy, apoptosis, colorectal cancer

**Introduction**

Colorectal cancer (CRC) is one of the most common gastrointestinal cancers with a relatively high mortality rate [1]. Contemporary, surgical measures are the primary option followed by chemotherapy in the CRC [2]. However, in spite of incredible advancement in treatment methodologies, the therapeutic outcome is insignificant due to acquired resistance and deleterious effects. The first line of therapy against CRC is 5-fluorouracil (5-FU) which persuades antitumor potential by mitigating the thymidylate synthase (TS) activity and reduces the synthesis of DNA and RNA [3]. Although 5-FU has established its survival benefits quite astonishingly, the applications in clinical settings have been significantly limited due to the development of drug resistance plus adverse side effects at higher doses [4]. Owing to poor clinical applications and toxicity issues, recent advancement has been applying novel combination therapeutic strategies to enhance the efficacy and reduce the adverse effects of drugs. The concept of combination therapy develops a new regime of the anti-tumor drug combination of CRC [5]. The current drug of choice for CRC is FOLFOX (5-FU, leucovorin with
Oxaliplatin or FOLFIRI (5-FU, leucovorin with irinotecan) in combination with bevacizumab, which boosts efficacy and enhances the survival rate of CRC patients with higher median survival period but the toxicity issue remains inevitable [6]. Thus, the need is to search for novel agents in combination with 5-FU for CRC patients is highly desired. Since natural products and their derivatives have the potential to combat drug resistance with minimum adverse effects, they are ideal for the combination therapy against CRC to enhance efficacy and reduce the toxicity of 5-FU.

Several natural compounds have been reported to exert antitumor effects by activating apoptosis through modulation of intracellular biological pathways involving ER stress and autophagy [7]. The accumulation of misfolded proteins within the ER lumen due to ER stress induces unfolded protein response (UPR). The UPR disrupts chaperone BiP (immunoglobulin heavy-chain binding protein GRP78) from all the three stress sensors PERK (protein kinase R (PKR)-like endoplasmic reticulum kinase), IRE1α (inositol-requiring enzyme 1α) and ATF6 (activating transcription factor 6) [8]. The activation of these stress sensors connects to specific cellular pathways within the cell and eventually culminates into apoptosis. The ER stress-associated apoptosis pathway is mediated by a key transcriptional factor called CHOP (C/EBP homologous protein) [9].

Autophagy is another biologically conserved catabolic process that involves degradation and recycling of old, damaged intracellular organelles and macromolecules such as proteins that are sequestered inside autophagosomes which eventually fuses with lysosomes for degradation [10]. Generally, under stress conditions, autophagy encourages cell survival. Although autophagy is conferred to be part of the resistance mechanism in the manner it helps in tumor cell survival [11]. However, autophagy may go either direction, which depends upon the stress and verdict regarding whether it can tolerate the stress severity to respond to survival mechanism or surrender to apoptosis due to the unbearable microenvironment [12]. Even if, autophagy favors cell survival during stress, simultaneous inhibition of autophagy and induction of apoptosis could progress the outcome in favor of the cancer treatment [13]. Several drugs or natural compounds promote autophagy by triggering cellular damage to DNA, proteins, and organelles. Such a class of drugs or natural compounds enhances rescindable damage to cells where autophagy plays a very crucial role in favor of therapeutics outcomes [14]. A plethora of evidence backs the notion that the tumor cells toggle between autophagy and apoptosis in a fine balance. This decisive transition plays a key role in the autophagic machinery and several proteins associated with autophagy tend to increase the induction and upregulation of apoptotic proteins under such stressful conditions [15]. Recent report insights that there is a link between the induction of ER stress-associated autophagy and apoptosis which represents a novel molecular axis for cancer therapeutics [16].

WA, a steroidal lactone, originally isolated from an Indian Ayurvedic medicinal plant traditionally called Ashwagandha or Withania somnifera, displays numerous pharmacological activities including anti-inflammatory, immunomodulatory, antitumor, pro-apoptotic, antiangiogenic, anti-fibrotic and anabolic activities [17-19]. Despite having several other biological activities, WA and its derivatives attenuate tumor growth by various mechanisms, including proteasome inhibition, p53 stabilization, reactive oxygen species (ROS) generation, induction of endoplasmic reticulum (ER) stress, apoptosis, inhibition of Akt phosphorylation and p38MAPK activation [20-24]. Additionally, WA exhibits anti-invasive, anti-metastatic properties and modulates epithelial to mesenchymal transition (EMT) and has attracted substantial preclinical analysis primarily for its anti-cancerous properties [25]. Recently, WA has been reported to induce autophagy, but it failed to kill breast cancer cells. However, we previously demonstrated that WA derivative 3-azido-withaferin A (3-AWA), not only inhibits invasion and motility but also induces autophagy and turns autophagic cells towards apoptosis in prostate apoptosis response-4 (Par-4) dependent manner in prostate cancer [26, 27]. Although WA has been reported to show the anticancer effect on CRC by various mechanisms, it is not yet certain whether WA induces ER stress-mediated autophagy and apoptosis.

In this study, we investigated whether a combination of WA and 5-FU induces autophagy and apoptosis via ER stress in CRC cells. Our results
Withaferin A and 5-fluorouracil induces cell killing in colon cancer cells revealed that combination not only promotes autophagy mode of cell killing but also induces apoptosis to reduce the cell viability and enhances the drug efficacy against CRC. Additionally, the combination effectively attenuates the hyperactivation of key proteins of the Wnt/β-catenin pathway by dephosphorylating β-catenin and GSK3β at Ser675 and Ser9 respectively and arrests the cell cycle in the G2/M phase. This catches the attention for the development of a novel combination of WA and 5-FU in CRC therapeutics.

Materials and methods

Cell culture and treatments

Human colorectal cancer cells WS480, HT-29, HCT-116 and normal colon cells NCM-460 were obtained from American Type Culture Collection (ATCC) and were cultured in the Dulbecco’s Minimal Essential Medium (DMEM) (#12100-061, GibCO) whereas NCM-460 normal colonic epithelial cells were cultured in M3 Base medium (#M300F, INCELL) containing 10% fetal bovine serum (FBS; Gibco/Invitrogen, #10270), penicillin-streptomycin solution (Invitrogen, #15070-063) and the humidified incubator with 5% CO2. All the cell lines were free from Mycoplasma contamination.

Chemicals, reagents, and antibodies

Acridine orange (AO; #A6014), 3-methyladenine (3MA; M9281), protease inhibitor cocktail (#S8820), phenylmethylsulfonyl fluoride (PMSF; #P7626), withaferin A (#W5394), dithiothreitol (DTT; #D9779), dimethyl sulfoxide (DMSO; #C6-164), 5-fluorouracil (5-FU; #F6625), Bradford reagent (#B6916), and annexin V and FITC (#APOAF) apoptosis detection kit were purchased from Sigma Aldrich. Ultracruz mounting medium (#SC-359850), Salubrinal (#SC-202332) and Z-VAD-FMK (#SC-3067) were procured from Santa Cruz Biotechnology. All antibodies were procured from Santa Cruz Biotechnology. All antibodies were procured from cell signalling technology; Poly (ADP-ribose) polymerase (PARP; #9542), β-actin (#4970), BiP (#3177), p-elF2α-ser51 (3398), CHOP (#2895), ATF-4 (#11815), p-PERK (#3179), LC3B-1/II (#3868), P62/SQSTM1 (#88588), Beclin1 (#3495), Caspase-3 (#14220), p-β-catenin-Ser675 (#9567), E-cadherin (#3195), p-GSK3β-Ser9 (#5558), vimentin (#12826), p-27 (#3686), p-21 (#29-47), Cyclin D (#55506), c-Myc (#18583), CDK4 (#12790), IRE1α (#3294), ATF-6 (#65880), and CDK2 (#2546). However, secondary antibodies, anti-mouse IgG (#SC-2005) and anti-rabbit (#SC-2357)-coupled with horseradish peroxidase (HRP) were procured from Santa Cruz Biotechnology.

Cell viability assay

Cell proliferation was analyzed by 3-(4, 5-dimethylthiazol-2-y1)-2, 5-diphenyltetrazolium bromide (MTT) assay as per the standard protocol [27]. Briefly, colon cancer cells (SW480, HT-19 and HCT 116) along with normal (NCM-460) cells were seeded at a density of 5×103 cells in each well of 96-well plate. The cells plated in triplicates were exposed to different concentrations of drugs (WA and 5-FU) alone or in combination for 24 h in a humidified incubator containing 5% CO2. After completion of incubation of the required time, cells were saturated with MTT dye (2.5 mg/ml) for 3 hrs at 37°C. The formazan crystals formed were dissolved in DMSO and the absorbance at 570 nm was determined using a microplate reader (Infinity M200). The cell viability percentage was determined according to the protocol described [27].

Combination index (CI) analysis

Drug interaction parameters such as dose-effect analysis, dose-response curves and the calculation of CIs were determined by CompuSyn software (CompuSyn, Inc., Paramus, NJ, USA). In general, if the drug interaction shows CI value less than 1, it implies synergism, if more than 1, suggests antagonism, whereas if the drug combination is equal to 1, indicates drug combination has an additive effect.

Detection of apoptosis

Apoptosis detection and quantification were analyzed by using the Annexin V and FITC apoptosis kit, as per the manufacturer’s instructions. CRC cells were plated at a density of 0.5×106 cells per well in a 6-well plate and exposed to different concentrations of drugs (WA and 5-FU) alone or in combination. After 24 h, cells were harvested, processed and stained with propidium iodide (PI) and annexin V as per manufacturer’s protocol. After completion of the staining process, cells were examined by
flow cytometry (BD FACS Aria II, BD Biosciences, San Jose) to quantify the apoptotic population.

**Detection of acidic vesicular organelles**

Acidic vesicular organelles were detected and quantified by AO dye using fluorescence microscopy and flow cytometry. Under normal conditions AO stains DNA and cytoplasm and appears green in color, however, in acidic compartments like autophagosomes, AO acts as an anisotropic fluorescent dye and fluoresces bright red in these compartments. Briefly, HCT-116 cells were exposed to different conditions of drugs (WA and 5-FU) alone or in combination for 24 h in the humidified CO₂ incubator at 37°C. After the completion of 2 h, cells were stained with AO (1 mg/ml) for 15 min, followed by thorough washing with PBS. The detection of AVOs which appears bright red under fluorescence was analyzed by fluorescence microscope LSM-510 (Carl Zeiss, Germany).

**Cell cycle analysis**

CRC cells at a density of 0.5×10⁶ were seeded in a 6-well plate and let them adhered to the surface overnight. Next morning cells were treated with different concentrations of drugs alone or in combination along with DMSO control for 24 h. After completion of the time point, cells were washed thoroughly with PBS and fixed with 70% ice-cold ethanol in -20°C overnight. Next day cells were processed for cell cycle analysis by incubating fixed cells with RNase (100 µg/ml) at 37°C for 30 minutes and stained with PI (50 µg/ml) in the dark for another 30 minutes at 4°C. The cell cycle analysis was performed by using the BDTM LSRII flow cytometry system (BD Bioscience, Franklin Lakes, USA).

**Phase-contrast microscopy**

Colon cancer cells (5×10⁴) were cultured on coverslips with different concentrations of the drug for 24 h to analyze the changes in the cellular morphology. After completion of the period, cells were processed for phase-contrast microscope LSM-510 (Carl Zeiss, Germany) to analyze the morphological changes in treated cells.

**Immunoblotting**

After overnight plating of 50×10⁵ cells/well in humidified 5% CO₂ incubator at 37°C, CRC cells were exposed with different concentrations of WA, 5-FU and combination along with DMSO vehicle. After 24 h, harvested cells were washed with ice-cold PBS, and were processed for lysis with lysis buffer containing (protease inhibitor cocktail, DTT 1 mM/L, NP-40 0.3%, HEPES 1 mM/L, sodium orthovanadate 1 mM/L, EDTA 1 mM/L, KCl 60 mM/L, PMSF 0.1 mM/L). The lysis solution obtained from cells was collected and processed at 4°C for centrifugation at a speed of 13000 g for 10 min. Supernatants collected from centrifugation in a separate tube were subjected to the Bradford method of protein estimation. The volume of cell lysis solution containing 20 µg of protein was calculated and subjected to SDS-PAGE from each sample treated with different drugs. After resolving properly, SDS-PAGE gel was transferred to the PVDF membrane (Millipore, IPVH00010), allowed it to incubate with a 5% (w/v) fat-free milk blocking solution containing 0.1% Tween-20 dissolved in PBS (Sigma, 274348). After blocking nonspecific proteins with a blocking solution, the PVDF membrane is incubated and probed with the desired antibodies (1:1000 dilutions) for at least 3 h at room temperature or overnight at 4°C. After incubation, the PVDF membrane is washed with TBST buffer three times, incubated and probed with species specific secondary antibodies tagged with horseradish peroxidase (Sigma, A6154 and Santa Cruz, SC-3697). Immunoblotting proteins were spotted by enhanced chemiluminescence (ECL) plus (Amersham, WBKLS0100).

**Real-time polymerase chain reaction (RT-PCR)**

Using Trizol reagent (Invitrogen, Carlsbad, CA), total RNA was extracted. RNA (1 mg) obtained from extraction, was reverse transcribed to synthesize first-strand cDNA by adding 100 units of Primer Script Reverse Transcriptase (Takara, 6110) and 50 mM Oligo Dt Primer (Takara, 6110). The cDNAs obtained were subjected to quantitative real-time PCR analysis. The sequences of the primers used were as follows: CHOP, 5'-ATGAGGACCTGCAAGAGGTCC-3' (sense) and 5'-TCCTCCTCAGTGCAAGCCAGC-3' (antisense); BiP, 5'-GTTCCTGCGCTGCAAGGTTG-3' (sense) and 5'-TGTTAGAGATGAACACTGCC-3' (antisense); PERK, 5'-CAGTGGGGATTGAGTGCTG-3' (sense) and 5'-GGAATGATCATCTTATTCCAA-3' (antisense); eIF2α, 5'-GCCTTTCTTGACTCTCTGGCACTC-3' (sense) and 5'-CCGTGTCCTTCTGGAAGG-3' (antisense); GAPDH, 5'-TGAACGGGGAAGCTCACTGG-3' (sense) and 5'-TCCACCAC-
CCTGGTGTCTGTA-3' (antisense). Using an ABI 7900HT Sequence Detection System (ABI Applied Biosystems, Foster City, CA), Real-time PCR was performed in a 10 mL reaction mixture which contains 1 mL cDNA template, primer 0.5 mM each, 5 mL SYBR Premix Ex Taq (Takara, RR420A), and ROX reference dye 0.2 mL. The PCR reaction was run for 30 sec at 95°C followed by 40 cycles of 95°C for 5 sec and 56°C for 30 sec. All the cDNA samples obtained were analyzed in triplicates. Endogenous control GAPDH was used for the entire experiment. The expression of gene analysis was performed using the comparative threshold cycle (2-ΔΔCT) method.

**siRNA knockdown assay**

PERK siRNA (sc-36213), IRE1α siRNA (sc-40705), and non-targeting siRNA (control siRNA) were obtained from Santa Cruz Biotechnology. However, ATF6 siRNA (EHU 015441) was from Sigma. Briefly, CRC cells (0.5×10⁵) were seeded in two 6-well plates (one for immunoblotting and another for annexin V FITC apoptosis detection) and transfected with different siRNAs (indicated above) using transfection reagent (T-2001-01) procured from DharmaFECT and used as per manufacturer’s protocol. After the 36 h post-transfection, cells were exposed to combination treatment (5-FU and WA) for 24 h. After the completion of 24 h, cells were harvested from both the plates and were processed for subsequent (immunoblotting and apoptosis) experiments.

**Statistical analysis**

All the experiments were accomplished for at least three independent times. The latest version of software Graph Pad Prism was used for statistical analysis of all independent unbiased experiments. The results obtained were denoted as the mean of ± SEM. Entire results were calculated by using the Student’s unpaired t-test, wherein a p-value of less than 0.05 was reflected significant (* means P < 0.05, **P < 0.01, and ***P < 0.001).

**Results**

The combination treatment induces synergistic anti-tumor effect by inhibiting CRC cell proliferation and induction of apoptosis

First, to examine the antiproliferative potential of 5-FU and WA, CRC cells (SW480, HT-29, HCT 116 cells) and normal colon NCM-460 cells were cultured and exposed to increasing concentrations of 5-FU and WA (0.1-100 µM) for 24 h. As demonstrated (Figure 1A and 1B), both 5-FU and WA significantly decreased the cell viability in a dose-dependent manner in CRC cells, and the 50% inhibitory concentrations (IC₅₀) of WA and 5-FU are in a range of (4.9 µM in SW480, 4.1 µM in HT-29, 3.7 µM in HCT 116) and (11.3 µM in SW480, 14.2 µM in HT-29, 4.7 µM in HCT 116) respectively. However, in non-malignant colon cells (NCM-460), the WA and 5-FU exhibited comparatively higher IC₅₀ values, 50 µM, and 46.2 µM respectively (Figure 1C), indicating that both the compounds had safe toxicity profile for normal colon cells at a concentration where they exerted an antiproliferative effect on CRC cells.

To investigate whether the combination treatment exerted any synergistic effect, a combination index (CI) was calculated by Compusyn software which allows us to determine whether the drug interaction shows synergism (CI < 1), antagonism (CI > 1) or additive effect (CI = 1). By using Compusyn software, A combination of WA (2.5 µM) and 5-FU (5.0 µM) exhibits a very strongest synergistic effect (Figure 1D). Additionally, the assigned combination doses of WA (2.5 µM) and 5-FU (5.0 µM) treatment exerts a significant antiproliferative effect in various CRC cells when compared with WA or 5-FU alone (Figure 1E).

For further confirmation of the antiproliferative effect of combination treatment, we performed Annexin V FITC assay after treating CRC cells with WA (2.5 µM), 5-FU (5.0 µM) and combination treatment along with DMSO as a control for 24 h. As shown in (Figure 2A) 33.5% cell population treated with combination were observed positive for Annexin V FITC staining (Figure 2B) and were significant compared to WA (12.6%) or 5-FU (8.2%) treatments. Further, poly ADP-ribose polymerase 1 (PARP1), quantified by immunoblotting analysis depicted (Figure 2C) prominent cleavage of PARP1 in a lane where cells were exposed to combination treatment. By phase-contrast microscopy, we observed a significantly higher number of dead, floating cells in combination treatment than WA or 5-FU alone treatment (Figure 2D), indicates that the activation of cell death pathways in treatment groups compared to DMSO control. To corroborate, the above results demonstrate that the combination treatment induces a potent antip-
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roliferative effect, exerts a very strong synergistic effect and pushes a significant population of CRC cells towards apoptosis.

Combination treatment promotes autophagy in CRC cells

In recent past WA has been reported to induce autophagy in breast cancer cells, however, the report reveals that WA fails to kill breast cancer cells [28]; the plausible explanation probably is due to high expression of Bcl-2 in breast cancer cells which eventually maintains firm interaction with Beclin1 and does not allow cells to go for programmed cell death type 2. Our previous report demonstrates that WA derivative, 3-azido-withaferin A (3-AWA) induces autophagy and turns autophagic cells towards apoptosis in Par-4 and concentration-dependent manner in the prostate cancer cells [26]. Therefore, we sought to reveal whether combination treatment could promote autophagy in CRC cells. To do this CRC cells were exposed to varying doses of drugs as depicted in Figure 3A. After completion of incubation for 24 h, immunoblotting of CRC cells in combination treatment revealed the prominent conversion of microtubule-associated protein 1 light chain 3 β-I (LC3B-I) to microtubule-associated protein 1 light chain 3 β-II (LC3B-II), which is a distinctive hallmark of autophagosome maturation assembly (Figure 3A). To check whether combination treatment affects the autophagy flux in CRC cells, we found high expression with the intense band
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Figure 2. A combination of WA and 5-FU induces apoptosis in CRC cells. A. CRC cells (SW480) were exposed to indicated concentrations of drugs (WA, 5-FU, and combination along with DMSO as a control) for 24 hrs and then subjected to Annexin V FITC and propidium iodide staining and analyzed by FACS. B. Bar diagram showing quantification of Annexin V FITC positive cells analyzed by FACS. C. Immunoblotting analysis showing prominent PARP cleavage in 4 lanes where cells were exposed to combination treatment and internal control β-actin. D. Representative micrograph images by phase-contrast microscopy of CRC cells (SW480) exposed to indicate doses of WA, 5-FU and combination treatment. The data represent the mean value ± SE of three independent experiments. ***P < 0.001.

intensity of sequestrosome 1 protein (P62), which is a specific substrate of autophagy, in combination treatment compared to WA or 5-FU treatment alone. However, the protein expression of another important sensor of the autophagy process, beclin1 did not change and remains consistently unchanged. Further, the study was commenced to inspect autophagic cells by acridine orange (AO) staining. As shown in Figure 3B and 3C, although, many cells in WA and 5-FU alone displayed AVOs treated cells the number of induction of prominent AVOs is significantly higher in combination treatment (Figure 3D) after quantification and appears bright red in the cytoplasm of CRC cells. Collectively, these results suggest that a combination treatment stimulates autophagic flux and augments cell death in CRC cells.

Combination treatment triggers ER stress in CRC cells

Growing evidence demonstrates that UPR-mediated ER stress and autophagy are dependent on each other and can lead to activation of autophagy [9], however, recently it has been reported that WA semi-synthetic derivative 3-AWA induces ER stress-mediated autophagy and turns on autophagic cells towards apoptosis at higher doses [26]. Thus, we wanted to determine whether the combination treatment could induce ER stress. As shown in Figure 4A, immunoblotting results revealed that there is a steady increase in expression of ER stress markers BiP, CHOP, ATF-4, and phosphorylation of p-eIF2α, p-PERK, however, the effect is more pronounced with combination treatment in CRC cells. Furthermore, pretreatment of salubrinal an ER stress inhibitor followed by combination treatment decreases the band intensity of BiP, CHOP, and ATF-4 and concomitantly reduces the phosphorylation of p-eIF2α and p-PERK. Additionally, immunocytochemistry results reveal that the intense fluorescence staining of CHOP protein in combination treatment than WA or 5-FU alone (Figure 4B). To confirm whether the ER stress induction by WA, 5-FU or combi-
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A combination treatment is at translational or at the transcriptional level, real-time PCR analysis reveal that mRNA expression of all ER stress markers (BiP, eIF2α, CHOP, and PERK) increases proportionately and the mRNA fold change expression is significantly higher after CRC cells were exposed to a combination treatment (Figure 4C and 4D). Collectively, these results suggest that combination treatment induces ER stress.

**Induction of apoptosis by combination treatment is dependent on the PERK axis and is independent on IRE1α or ATF-6 axis of ER stress**

The ER stress-mediated UPR activates apoptosis and cell survival signals simultaneously and then promotes pro-apoptotic or cytoprotective functions. The ER stress executes survival and adaptation processes when the stress is mild, while activates the proapoptotic axis when the ER stress is severe [29, 30]. In the recent past, reports suggest that depending upon the condition and severity of ER stress, different pathways are activated selectively. Among the ER stress signaling pathways, the PERK axis plays a major role in the induction of ER stress-mediated apoptosis and attenuates cellular growth in various tumor cell lines [31, 32].

To explore, which axis of ER stress signaling pathways plays a crucial role in the induction of apoptosis by combination treatment, we transiently transfect CRC cells with control siRNA and siRNAs of all the three main axis players of ER stress sensors: PERK, IRE1α, and ATF-6. After the 36 h of post-transfection, CRC cells
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were exposed to combination treatment for the next 24 h. The cells were processed, subjected to immunoblotting and annexin V FITC staining to analyze the expression and quantification of apoptotic cells. The immunoblotting data revealed that the transfection of CRC cells with PERK siRNA followed by combination treatment for 24 h, had a feeble amount of expression of pro-apoptotic protein CHOP with the concomitantly negligible expression of cleaved PARP1 and caspase-3 Figure 5A. Interestingly, knockdown of IRE1α and ATF-6 genes with respective siRNAs followed by combination treatment increases the expression of pro-apoptotic protein CHOP with subsequent activation and cleavage of PARP1 and caspase-3 when compared to PERK knockdown cells followed by combination treatment Figure 5A. For further confirmation, quantification of apoptosis by annexin V FITC revealed that PERK knockdown cells followed by combination treatment had a significant reduction in apoptosis population (3.6%) than cells exposed to combination treatment only (31.7%). However, IRE1α and ATF-6, knockdown cells followed by combination treatment have apoptotic population (IRE1α + combination - 30.6% and ATF-6 + combination - 30.0%) as good as that of combination treatment only (Figure 5A and 5C). Collectively, these observations demonstrated that PERK knockdown by respective siRNA could not restore the expression of ER stress-associated proapoptotic protein CHOP and apoptotic population of cells even in presence of combination treatment and verifies that ER stress-mediated apoptosis induction by combination treatment is operated via PERK axis in CRC cells.
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Induction of autophagy and apoptosis is dependent on ER stress-mediated combination treatment and could not interconvert when challenged with pharmacological inhibitors

We next sought, whether; combination treatment-mediated ER stress was associated with autophagy and apoptosis. Firstly, immunoblotting results revealed that combination treatment increases the expression of ER stress sensor CHOP and phosphorylation of p-eIF2α, p-PERK (Figure 6A). Besides ER stress markers, under the same, combination treatment effectively augments apoptosis markers PARP-1, and caspase-3 cleavage in CRC cells (Figure 6A), we also noticed a steady conversion of LC3-I to LC3-II and prominent band intensity of p62 a sequestosome proteins involved in the autophagosome maturation process. Secondly, the abrogation of ER stress by salubrinal inhibits phosphorylation of ER stress sensor p-eIF2α, p-PERK and decreases the expression of CHOP proteins. Unexpectedly, our results revealed that ER stress inhibition also blocks maturation of autophagosome formation (AVOs) (Figure 6A and 6B) as noticed from immunoblotting the decrease in conversion of LC3-I to LC3-II and p62 protein expression, moreover, decrease in cleavage of PARP1 and caspase-3 (Figure 6B) reduces apoptotic population of cells, indicates attenuation of apoptosis due to inhibition of ER stress.

Autophagy and apoptosis are two interrelated and biologically conserved processes [33]; we next determine whether combination treatment mediated autophagy and apoptosis could interconvert in one another if challenged pharmacologically. To do that, CRC cells were exposed to

Figure 5. Combination induced apoptosis in CRC cells is dependent on PERK axis and is independent on IRE1α or ATF-6 axis of ER stress: (A) Immunoblotting analysis of knockdown CRC cells with PERK, IRE1α and ATF-6 siRNAs for 36 h, followed by combination treatment for 24 h, along with control siRNA and combination treatment alone shows the expression of ER stress proteins (CHOP, p-PERK, IRE1α, and ATF-6), and apoptosis (PARP1 and caspase-3) markers along β-actin as internal control. (B) CRC (HCT116) cells were transiently transfected with siRNAs to knockdown all the major three axis of ER stress sensors: PERK, IRE1α and ATF-6 and followed by combination treatment for 24 h, along with control siRNA and combination treatment alone. The cells were processed, subjected to Annexin V FITC and propidium iodide staining and analyzed by FACS. (C) Bar diagram showing quantification of Annexin V FITC positive cells analyzed by FACS under different settings. The data represent the mean value ± SE of 3 independent experiments. **P < 0.01.
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Figure 6. Induction of autophagy and apoptosis is dependent on ER stress-mediated by the combination of WA and 5-FU in CRC cells. A. Immunoblotting analysis of CRC cells (HCT 116) exposed to various drug concentrations (WA or 5-FU alone and in combination) in absence or presence of Saubrinal shows the expression of ER stress (CHOP, p-eIF2α, p-PERK), autophagic (P62, LC3-I/II) and apoptosis (PARP1 and caspase-3) markers along β-actin as internal control. B. Immunoblotting analysis of CRC cells (HCT 116) exposed to various drug concentrations (WA or 5-FU alone and in combination) in absence or presence of pharmacological inhibitors (3-MA and Z-VAD-FMK) shows the expression of ER stress (CHOP, p-eIF2α, p-PERK), autophagic (P62, LC3-I/II) and apoptosis (PARP1 and caspase-3) markers along β-actin as internal control. Immunostaining of CHOP protein by fluorescent microscopy, after SW480 cells were exposed to indicate the concentration of WA, 5-FU alone or in combination. C and D. The bar diagram presents the quantification of the AO positive and apoptotic cell population in presences of pharmacological inhibitors (Salubrinal, 3-MA, and Z-VAD-FMK). The data represent the mean value ± SE of 3 independent experiments. ***P < 0.001.

WA, 5-FU alone or combination treatment in the presence and absence of autophagy inhibitor 3-MA and apoptosis inhibitor Z-VAD-FMK (pan-caspase inhibitor). Our results showed that pre-treatment of cells with 3-MA followed by combination treatment, inhibits expression of autophagy protein P62 and LC3-I to LC3-II but not as effectively as 3-MA alone, next we try to investigate whether the 3-MA mediated autophagy inhibition in combination treatment could turn on apoptosis, our results reveal that the slight reduction in band intensity of PARP1 and caspase-3 cleaved products compared to combination treatment (Figure 6C). AO staining and apoptosis quantification reveal a significant reduction in AO positive cells and feeble reduction in the apoptotic cell population respectively. Next, Z-VAD-FMK pretreated CRC cells exposed to combination treatment completely decrease the cleavage product of PARP1 and caspase-3, however, no significant change in band intensity of autophagic proteins P62 and LC3-I to LC3-II were observed (Figure 6C and 6D). Moreover, quantification of AO positive and apoptotic cells reveals that Z-VAD-FMK drastically decreases the apoptotic population,
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Whereas no such significant change was observed in AO positive stained cells. Collectively, these findings demonstrate that inhibition of autophagy or apoptosis by their respective pharmacological inhibitors in the presence of combination treatment could not interconvert autophagic cells in apoptosis.

Combination inhibits β-catenin signaling pathways and simultaneously triggers cell cycle arrest at a G2/M phase in CRC cells

The Wnt/β-catenin signaling pathway is hyper-activated in CRC cells. We next examine the effect of combination treatment on Wnt/β-catenin signaling pathways. Immunoblotting analysis revealed that the decrease in the phosphorylation of β-catenin (Ser675) and GSK3β (Ser9) in combination-treated CRC cells, further combination treatment shows a prominent increase in E-cadherin expression and downregulation of the vimentin expression (Figure 7A). Next, we sought to determine whether inhibition of β-catenin with combination treatment could have an impact on cell cycle proteins. To do that CRC cells were exposed to indicate concentrations of drugs. Our results revealed that combination treatment reduces the expression of cyclin D1, CDK4, CDK2, c-Myc, p21/Cip1, and p27/Kip1 along β-actin as an internal control. C and D. The bar diagram presents the quantification of CRC cells in different stages of the cell cycle after exposing them to various drug concentrations (WA or 5-FU alone and in combination). The data represent the mean value ± SE of 3 independent experiments. ***P < 0.001.

**Figure 7.** A combination of inhibits β-catenin signaling pathways and arrests cell cycle in CRC cells. A. Immunoblotting analysis of CRC cells (HCT 116) exposed to various drug concentrations (WA or 5-FU alone and in combination) shows the expression pattern of β-catenin and associated proteins (E-cadherin, Vimentin, and GSK3-β) along β-actin as an internal control. B. Immunoblotting analysis of CRC cells (HCT 116) exposed to various drug concentrations (WA or 5-FU alone and in combination) shows the expression profile of cell cycle regulation proteins (cyclin D1, CDK4, CDK2, c-Myc, p21/Cip1, and p27/Kip1) along β-actin as an internal control. C and D. The bar diagram presents the quantification of CRC cells in different stages of the cell cycle after exposing them to various drug concentrations (WA or 5-FU alone and in combination). The data represent the mean value ± SE of 3 independent experiments. ***P < 0.001.
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Discussion

The significant advancement in the therapeutics of colorectal cancer (CRC) by using various promising drugs such as oxaliplatin, 5-FU, and antibodies like bevacizumab and cetuximab which kills cancer cells by inducing programmed cell death mechanisms to improve the overall survival rate of patients [34, 35]. However, the swift emergence of drug resistance and adverse effects diminishes the efficacy of conventional chemotherapy [36]. Recent advancement reveals that combinatorial therapy has numerous advantages over conventional therapy and represents a promising approach to overcome the deleterious effects by reducing the drug dosage [37]. Despite the remarkable achievement in overall survival rate in CRC patients with combination therapy in the recent past, however, the toxicity issue cannot be circumvented completely and still makes inroads in the path of combination therapy. Therefore, the need to search for more specific combination therapies with less deleterious effects on normal cells warrants the highest importance. Since plant-derived natural products are complex, they displayed tremendous pharmacological activities and yielded various promising anticancer lead molecules for novel anticancer drug discovery. Owing to the least side effects on normal cells, natural products used in combination with approved drug 5-FU is an ideal approach to kill CRC cells more specifically and benefit patients to achieve better therapeutic outcomes and increase the quality of life. Here, in this study, a promising anticancer natural product compound WA in combination with 5-FU is subjected to evaluate the antitumor synergistic effect in CRC cells to improve outcomes in terms of efficacy of the drug combination by decreasing the dosage level and reduce adverse effects on normal cells.

In this study, our detailed analysis demonstrated that the combination treatment drastically decreases cell viability in CRC cells and exerts a very strong combination index, which not only reduces the dosage level of respective combination but also reveals strong efficacy and safe toxicity profile in normal colon cells. Further, results showed that the combination treatment induces UPR driven ER stress, which activates two interrelated and crosses connected biological pathways which help in the efficient killing of tumor cells both by programmed cell death I (apoptosis) and II (autophagy). Further, owing to the hyperactivation of β-catenin/Wnt pathway in CRC cells, combination treatment attenuates the phosphorylation of key proteins of β-catenin/Wnt signaling which eventually triggers cell cycle arrest at G2/M phase and enhances apoptosis of CRC cells, suggesting that the combination treatment exerts a very strong anti-tumor synergistic effect on CRC cells.

We examined the effect of combination treatment on cell viability of CRC cells. The combination treatment drastically decreases cell viability at lower doses compared to WA or 5-FU alone. Intriguingly, drug index analysis showed that combination treatment induces a very strong synergistic index, increases drug efficacy and displays a safe toxicity profile in normal colon cells. A similar study conducted by Ambroz M et al. demonstrated that sesquiterpene derived from Myrica rubra increases the efficacy of doxorubicin in both sensitive and resistant cancer cells [38]. Recently another study showed that pullulan-encapsulated lovastatin (PLV) nanoparticles increase the efficacy of doxorubicin against triple-negative breast carcinoma (TNBC) cells [39]. Likewise, nanoeulsion (NE)-loaded particles decrease the IC_{50} concentration of paclitaxel and dual inhibitor of PI3K/mTOR pathway, BEZ235 by increasing efficacy and enhance cell killing in CRC cells [40].

In the recent past, it has been reported that many potential antineoplastic drugs activate cell death mechanisms by inducing unfolded protein response (UPR)-mediated ER stress in tumorigenic cells and modulates ER homeostasis [41]. The modulation of ER homeostasis results in dissociation of membrane-associated proteins; PERK, IRE1 and ATF-6 and accumulation of chaperone protein (BiP) in the ER lumen [42]. Depending upon the stress severity, cancer cells make decision making calls, whether to restore protein unfolding or to acti-
vate the cell death mechanisms via autophagy or apoptosis [9]. The temporary attenuation of protein translation and transcription of chaperone associated genes in mild ER stress restores the protein folding in cells [43], however, failure of resolving ER stress activates two interconnected biological processes viz: autophagy and apoptosis [44]. On the one side, the accumulation of misfolded proteins is degraded by autophagy mechanisms and on the other side [45], the severity of ER stress phosphorylates PERK-mediated eIF2α which further activates the ATF4 a transcriptional factor and CHOP a key regulator of ER-associated apoptosis [46]. Consistent with recent data, the current study exerts a synergistic antitumor effect by activating ER stress-mediated autophagy and apoptosis. Subsequent analysis revealed that the combination treatment in CRC cells not only disrupts the association of ER sensors but also increases the protein expression of BiP, CHOP, ATF-4 and augments the phosphorylation of PERK and eIF2α. Additionally, knockdown of all the three major axis players of ER stress sensors: PERK, IRE1α, and ATF-6 by respective siRNAs, suggest that combination treatment-induced apoptosis is operated through the PERK axis of ER stress in CRC cells. Further, the analysis showed that the expression of ER stress sensors increases at the transcription level and consistent with previous data suggest that induction of apoptosis in combination treatment cells is mediated by ER-associated pro-apoptotic axis (PERK/eIF2α/CHOP). Additionally, pretreatment with the ER stress inhibitor salubrinal drastically decreases the autophagy and apoptosis in the combination treatment, suggests that subsequent activation of autophagy and apoptosis in CRC cells exposed to combination treatment is dependent on ER stress.

Hyperactivation due to mutations or deregulation causes β-catenin upregulation in many cancers, particularly in CRC and causes tumor progression, survival, and metastasis [46]. Recent findings revealed that β-catenin expression increases during the progression of the cell cycle and reaches the optimum level in the G₂M phase of the cell cycle [47]. Another finding demonstrated that pancreatic cancer cells exposed to withanolide-D, a steroidal lactone, abrogates the β-catenin signaling pathway and triggers arrest of the G₂M phase of the cell cycle [48]. A key component of the canonical Wnt-signalling pathway, β-catenin plays a crucial role in developmental processes and other diverse biological processes including cell growth, differentiation, survival, invasion, migration, polarity and cell stemness [49]. The immediate downstream target of the Wnt/β-catenin signaling pathway, c-Myc plays a key role in the rearrangement of chromosomes, telomere remodeling, and arrest of the cell cycle at G₂M phase during DNA damage and allows incorrect entry of impaired chromosomes into mitosis [50]. Intriguingly, more than 60% of CRC patients have deregulated c-Myc expression and are implicated in the development of CRC and inflicted in chemotherapeutic resistance against 5-FU [51, 52]. Consistent with the previous study of pancreatic adenocarcinoma cells exposed to withanolide D, our findings strongly indicate that combination treatment abrogates phosphorylation of Ser675 of β-catenin and Ser9 of GSK3β a key mediators of Wnt/β-catenin pathway, subsequently, the E-cadherin and vimentin expression increases and decreases respectively. Furthermore, combination treatment in CRC cells decreases the expression of c-Myc, cyclin D, CDK4, CDK2 and concomitantly augments the protein expression of cell cycle checkpoints p21/Cip1 and p27/Kip1, eventually triggers arrest at G₂M phase of the cell cycle.

In summary, the current study demonstrated that combination treatment showed a strong antiproliferative effect in CRC cells at low concentration and exerts very strong combination index, which not only decreases the dosage level of respective combination but also reveals strong efficacy and safe toxicity profile in normal colon cells. Mechanistically, combination induces ER stress-mediated induction of autophagy and apoptosis to augment cell death mechanisms in CRC cells (Figure 8). Besides, it prevents hyperactivation of the Wnt/β-catenin pathway by dephosphorylating key mediators β-catenin and GSK3β at positions Ser675 of Ser9 respectively, combination treatment enhances protein expression of checkpoint regulators (p21/Cip, p27/Kip) and triggers cell cycle arrest of G₂M phase of the cell cycle. The aim of using natural products in such types of combination studies is to reduce side effects and enhance the efficacy of approved drugs against anticancer therapeutics.
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Disclosure of conflict of interest

None.

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References


Figure 8. Schematic diagram showing a possible antitumor mechanism of the combination treatment of 5-FU and WA.


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