World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v19.i42.7374

World J Gastroenterol 2013 November 14; 19(42): 7374-7388
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

1,3-Bis(2-chloroethyl)-1-nitrosourea enhances the inhibitory effect of Resveratrol on 5-fluorouracil sensitive/resistant colon cancer cells

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Supported by Indian Council of Medical Research and Department of Biotechnology, Government of India
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Received: December 19, 2012 Revised: April 25, 2013
Accepted: June 5, 2013
Published online: November 14, 2013

Abstract

AIM: To study the mechanism of 5-fluorouracil (5-FU) resistance in colon cancer cells and to develop strategies for overcoming such resistance by combination treatment.

METHODS: We established and characterized a 5-FU resistance (5-FU-R) cell line derived from continuous exposure (25 μmol/L) to 5-FU for 20 wk in 5-FU sensitive HCT-116 cells. The proliferation and expression of different representative apoptosis and anti-apoptosis markers in 5-FU sensitive and 5-FU resistance cells were measured by the MTT assay and by Western blotting, respectively, after treatment with Resveratrol (Res) and/or 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU). Apoptosis and cell cycle arrest was measured by 4',6'-diamidino-2-phenylindole hydrochloride staining and fluorescence-activated cell sorting analysis, respectively. The extent of DNA damage was measured by the Comet assay. We measured the visible changes in the DNA damage/repair cascade by Western blotting.

RESULTS: The widely used chemotherapeutic agents BCNU and Res decreased the growth of 5-FU sensitive HCT-116 cells in a dose dependent manner. Combined application of BCNU and Res caused more apoptosis in 5-FU sensitive cells in comparison to individual treatment. In addition, the combined application of BCNU and Res caused a significant decrease of major DNA base excision repair components in 5-FU sensitive cells. We established a 5-FU resistance cell line (5-FU-R) from 5-FU-sensitive HCT-116 (mismatch repair deficient) cells that was not resistant to other chemotherapeutic agents (*e.g.*, BCNU, Res) except 5-FU. The 5-FU resistance of 5-FU-R cells was assessed by exposure to increasing concentrations of 5-FU followed by the MTT assay. There was no significant cell death noted in 5-FU-R cells in comparison to 5-FU sensitive cells after 5-FU treatment. This resistant cell line overexpressed anti-apoptotic [*e.g.*, AKT, nuclear factor κB, FLICE-like inhibitory protein), DNA repair (*e.g.*, DNA polymerase beta (POL-β), DNA polymerase eta (POLH), protein Flap endonuclease 1 (FEN1), DNA damage-binding protein 2 (DDB2)] and 5-FU-resistance proteins (thymidylate synthase) but under expressed pro-apoptotic proteins (*e.g.*, DAB2, CK1) in comparison to the parental cells. Increased genotoxicity and apoptosis were observed in resistant cells after combined application of BCNU and Res in comparison to untreated or parental cells. BCNU increased the sensitivity to Res of 5-FU resistant cells compared with parental cells. Fifty percent cell death were noted in parental cells when 18 μmol/L of Res was associated with fixed concentration (20 μmol/L) of BCNU, but a much lower concentration of Res (8 μmol/L) was needed to achieve the same effect in 5-FU resistant cells. Interestingly, increased levels of adenomatous polyposis coli and decreased levels POL-β, POLH, FEN1 and DDB2 were noted after the same combined treatment in resistant cells.

CONCLUSION: BCNU combined with Res exerts a synergistic effect that may prove useful for the treatment of colon cancer and to overcome drug resistance.

Key words: 5-fluorouracil; 1,3-Bis(2-chloroethyl)-1-nitrosourea; Resveratrol; Colon cancer; Combination therapy

Core tip: 5-fluorouracil (5-FU) resistance in colon cancer patients is a common phenomenon that requires immediate resolution. In this paper, we used two commonly administered clinical drugs, 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) and Resveratrol (Res), and studied their effects on 5-FU sensitive and resistance colon cancer cells. The drug combination was more effective in 5-FU resistant cells than in 5-FU sensitive cells and was more effective than the individual treatments. The BCNU-Res combination mediated its action by compromising the base excision repair cascade. This combination might be useful for treatment of 5-FU resistant colon cancer patients.

Das D, Preet R, Mohapatra P, Satapathy SR, Kundu CN. 1,3-Bis(2-chloroethyl)-1-nitrosourea enhances the inhibitory effect of Resveratrol on 5-fluorouracil sensitive/resistant colon cancer cells. *World J Gastroenterol* 2013; 19(42): 7374-7388 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i42/7374.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i42.7374

INTRODUCTION

Colon cancer ranks as second in cancer related deaths in Western countries, and accounts for approximately 10%-15% of all cancers; half of these patients eventually metastasize[1]. The prognosis for patients who develop metastatic tumors is very poor, although several chemotherapeutic methods have been used to improve their survival and quality of life[2]. The antimetabolite 5-fluorouracil (5-FU) has been used as a first line therapy in colon cancer; however, in the clinical setting, more than 40% of cases are resistant. Combinations of 5-FU with Leucovorin (LV), Cisplatin, and Oxaloplatin, or with plant derived compounds including Curcumin, Resveratrol, and Quinacrine have increased the response rate up to 25%-30%[3-8]. 5-FU exerts anti-proliferative and cytotoxic effects on cells by inhibiting thymidylate synthase (TS) or by misincorporation into DNA and RNA[9].

The misincorporation of 5-fluorodeoxyribosyl residues in DNA is generally repaired by mismatch repair (MMR)[10-12] and cells grow continuously. However, any disturbance of the repair system will cause incomplete removal of 5-FU, leading to cell death. Thus, resistance to 5-FU in certain cancers may be the result of highly efficient DNA repair in the 5-FU resistant cells. Several investigators have used multiple combinations of drugs to increase the sensitivity of 5-FU resistant-cancer cells, but these studies showed very poor clinical responses[4-6,8].

The well-known natural product Resveratrol (Res) has demonstrated high antitumoral efficiency without harmful side effects of conventional chemotherapies. Res (3,4,5-trihydroxy-trans-stilbene) is a polyphenolic phytoalexin widely present in plants and enriched in red grapes, peanuts and other sources[13]. This compound demonstrates beneficial functions in normal cells both in *in vitro* and *in vivo* model systems[13]. On the other hand, this compound exhibits cytotoxic effects on the majority of malignant cells, blocking the three major stages of carcinogenesis (*i.e.*, initiation, promotion and progression)[13] in several cancer cell types, such as breast, colon, melanoma, uterine, lung and leukemia cells[13-21]. Recently, using HCT-116 colon cancer cells, we demonstrated that 5-FU increases the sensitivity to resveratrol by inducing DNA damage and the MAPK pathway[7]. A significant inhibition of cell proliferation, migration, and increased apoptosis were observed when moderate concentration of Res (15 μmol/L) were combined with very low concentration of 5-FU (0.5 μmol/L)[7].

1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU or carmustine) belongs to the family of haloethylnitrosoureas. It is a common synthetic alkylating chemotherapeutic agent used for treating various types of cancers, including brain tumors, Hodgkins and non-Hodgkins lymphoma and multiple myeloma[22]. It is highly lipophilic, capable of readily crossing the blood-brain barrier. It reacts with DNA and forms several exocyclic DNA adducts, which includes the saturated ethanol adducts of adenine, cytosine, and guanine (1,*N*6-ethanol-A, 3,*N*4-ethanol-C, *N*2,3-ethanol-G, and 1,*O*6-ethanol-G) and produces intra strand cross-links in DNA[23-26]. It can also produce mono substituted purine bases in DNA[23]. BCNU is classified as an animal carcinogen[27], and has been demonstrated as genotoxic both *in vivo* and *in vitro*[27]. This limits the use of this drug at higher doses, unless the drug is combined with some natural anticancer compounds that will not only decrease the toxic effect, but also synergistically increase its effectiveness. Several combinations of BCNU have been tried, such as with As_2O_3[28], O^6BG[29] and TMZ[30], to increase the sensitivity in cancer cells, but without success.

Recently, researchers attempted to target DNA damage/repair and cell cycle checkpoint signaling pathways as a means of cancer treatment[31-33]. This is relevant for 5-FU therapy, as TS inhibition and incorporation of the fluorinated base into DNA occurs during S-phase. The PI3K-like kinases, ataxia telangiectasia-mutated (ATM) and ATM-related (ATR), are central mediators in the response to DNA damage during S-phase[34]. Evidence suggests the involvement of S-phase checkpoint pathways in response to 5-FU treatment and TS inhibition[35]. A recent report suggested that ATR and CHK1 status influence cellular sensitivity to 5-FU in a MMR- or BER-mediated response-dependent manner, dictated by the

drug dose and exposure period[36]. It was also reported that 5-FU-mediated apoptosis also involved modulation of the major long patch base excision repair (LP-BER) protein Flap endonuclease 1 (FEN1)[10,37]. We have already reported that activation of adenomatous polyposis coli (APC) blocks the base excision repair pathway by interaction with DNA polymerase beta (POL-β) and FEN1[38-41]. Thus, one strategy for killing 5-FU resistant cells includes combining multiple drugs that will damage DNA, inhibit the DNA repair system and hamper cell cycle regulation in cancer cells.

In the present study, we developed a novel chemotherapeutic combination to increase the sensitivity of 5-FU resistant colon cancer cells using a synthetic DNA damaging agent, BCNU, and a plant derived anti-cancer agent, Res, and investigated the mechanism of anti cancer potentiality against 5-FU resistance. We first measured the anti-cancer potentiality of this combination in 5-FU sensitive human colon cancer cells. A 5-FU resistant cell line was derived from HCT-116 colon cancer cells after prolonged treatment with 5-FU, which was sensitive to other chemotherapeutic agents. These cells were treated with the BCNU and Res combination and drug efficacy was measured. The BCNU and Res combination increased apoptosis of the 5-FU resistant cell lines by inducing DNA damage and inhibiting the base excision repair pathway. We also used isobologram analysis to evaluate whether the drug combination has a synergistic, additive or antagonist effect on human colon cancer.

MATERIALS AND METHODS

Cell culture and treatment

The HCT-116 colon cancer cell line (obtained from American Type Culture Collection, VA, United States, Cat # CCL-247) was cultured in RPMI-1640 with 1% antibiotic (100 units of penicillin and 1 mg streptomycin per milliliter in 0.9% normal saline) and supplemented with 10% fetal bovine serum (HIMEDIA, Mumbai, India) in a humidified CO_2 incubator in 5% CO_2 at 37 ℃. Drugs like Res, 5-FU, LV, BCNU were purchased from Sigma Chemical Ltd. (St. Louis, MO, United States). DNA polymerase eta (POLH) and DNA damage-binding protein 2 (DDB2) antibodies were procured from Abcam (MA, United States). Apurinic/apyrimidinic (AP) endonuclease 1 (APE) and POL-β antibodies were purchased from Novus Biologicals (CO, United States). The anti-APC antibody was purchased from Calbiochem (CA, United States). All the other antibodies used in the experiments were procured from Cell Signaling Technology (Danvers, MA, United States). A 1 mmol/L stock of Res, 5-FU, BCNU and LV was prepared in absolute ethanol, DMSO, 50% ethanol and distilled water, respectively, and was stored at -20 ℃. During treatment, Res, 5-FU and BCNU were diluted in RPMI and then added to the cultures to achieve the desired final concentration. LV (1 μmol/L) was added in combination with 5-FU in each treatment. When they reached 60%-70% confluency, the cells were treated with 5-FU, Res, BCNU and the combination of BCNU and Res (in each case of the combination treatment, the cells were pre-treated with BCNU (20 μmol/L) for 24 h and then exposed to Res for another 48 h).

Establishment of 5-FU resistant cell line

In our earlier report, we demonstrated that the minimum concentrations needed for causing fifty percent cell death in culture (LC_{50}) in HCT-116 cells by 5-FU was 10 μmol/L[7]. To make stable 5-FU resistant cell lines, HCT-116 cells were exposed to 25 μmol/L for more than 20 wk. Every 48 h the media was replaced with fresh media and cells were treated with fresh 5-FU of same concentration. Initially, more than 80% of the cells died within a few days; the remaining 20% cells survived and developed 5-FU resistance. At the end of the exposure, 5-FU resistance was checked by a cell survival assay (MTT) in the presence of 5-FU and the transformed characteristic was confirmed by measuring several biomarkers. No significant cell death was noted after 5-FU treatment allowing the cells to be designated as 5-FU-R cells.

MTT assay

The anchorage dependent cell viability of normal HCT-116 and 5-FU-R cells was measured after treatment with various drugs using a reliable and sensitive colorimetric assay: the MTT [3-(4,5-Dimethylthiazol-2yl-)-2,5-diphenyl tetrazolium bromide] cell proliferation assay, as described previously[7,42]. Approximately, 10000-12000 cells were plated in a 96 well tissue culture plate in triplicate and incubated for 24 h, after which they were exposed to various concentrations of the indicated compounds for 48 h. At the end of the treatment, the cells were washed with 1 × phosphate buffered saline (PBS) and then 100 μL of 0.05% MTT reagent (Sigma) was added to each well and kept for 6 h in incubation at 37 ℃ to allow the formation of purple formazan crystals. 100 μL of detergent solution (10% NP-40 with 4 mmol/L HCl) was added to each well of the 96 well tissue culture plate and the reaction mixture was incubated for 1hr at room temperature. The color intensity was measured spectrophotometrically using a microplate reader (Berthold, Germany) at 570 nm. The data were calculated as percentages of the control. All assays were performed at least three times.

Nuclear staining with 4',6-diamidino-2-phenylindole

To determine the apoptosis of HCT-116 and 5-FU-R cells after treatment with the indicated drug, 4',6-diamidino-2-phenylindole (DAPI) nuclear staining was carried out. Cells at 70%-80% confluency were treated with the desired drug for indicated time, as mentioned in the figure legend. The cells were then washed with PBS and fixed with acetone: methanol (1:1) for 15 min at -20 ℃ in the dark. Fixed cells were washed once with PBS and then DAPI solution was added and incubation was continued for 1 h at 37 ℃ in the dark. Excess DAPI was removed by washing with PBS and the stained cells were visualized under a fluorescence microscope (Nikon, Japan) at × 40 magnification.

Cell cycle

Regulation of cell cycle and apoptosis were measured using fluorescence-activated cell sorter (FACS) analysis. HCT-116 and 5-FU-R cells were cultured in 60 mm tissue culture discs until they reached 60%-70% confluency. They were then treated with the indicated drug. After treatment, cells were trypsinized and washed with PBS containing RNase-A and fixed with 70% ethanol. The cells were incubated at -20 ℃ overnight and then re-suspended in 0.1 mL of Propidium Iodide (PI, 50 μg/mL) and incubated for 1 h in the dark at room temperature. Finally, FACS was used to sort the cells (Becton and Dickinson, CA). Cell Quest Software was used to determine the DNA content of the cells at various phases of the cell cycle (Becton and Dickinson, CA).

Single cell gel electrophoresis or comet assay

After drug treatment, DNA damage in HCT-116 and 5-FU-R cells was determined by performing a single cell gel electrophoresis assay following the method of Tice *et al*[43]. Cells were suspended in PBS and about 5000-6000 cells were mixed with low melting agarose at 37 ℃ and spread on a microscopic slide. After solidifying, the slides were dipped in chilled lysis solution (10 mmol/L Tris, 100 mmol/L EDTA, 2.5 mol/L NaCl, 1% Triton × 100, 10% DMSO and pH 10.0) for 1 h. After lysis the slides were put in freshly prepared alkaline electrophoresis buffer and electrophoresis was performed at 20 V for 20 min. Slides were then dipped in neutralization buffer (0.4 mol/L Tris-HCl and pH 7.5) and washed with distilled water, followed by 70% ethanol, and allowed to dry. Later, 40 μL of SYBR green dye was added to each slide, which was incubated in the dark for 30 min at room temperature. A fluorescence microscope (Nikon, Japan) at a magnification of × 10 was used to visualize DNA migration. TriTek CometScore™ software was used to analyze the comet lengths.

Western blotting

Cells were plated on 100 mm tissue culture discs and were treated with the indicated drug when they reached 70% confluence. After treatment, the cells were washed with PBS and were lysed using the RIPA lysis buffer (50 mmol/L Tris, 150 mmol/L NaCl, 0.5 mmol/L Deoxycholate, 1% NP-40, 0.1% SDS, 1 mmol/L Na_3VO_4, 5 mmol/L EDTA, 1 mmol/L PMSF, 2 mmol/L DTT, 10 mmol/L β-glycerophosphate, 50 mmol/L NaF, 0.5% Triton × 100, protease inhibitor cocktail) for 45 min at 4 ℃. The lysates were centrifuged at 14000 rpm for 10 min to remove the cell debris. The supernatant was collected in a separate tube and protein quantification was performed by Bradford method using BSA as a standard. Approximately 100 μg of protein were loaded in each well and separated using 10% SDS-PAGE. After separation, the proteins were transferred to a PVDF membrane. The membrane was blocked and then probed with specific antibody, according to the manufacturer's protocol.

Analysis of combined drug effect

Synergistic, additive or antagonist drug effects were determined by isobologram analysis[28]. Isobologram plots were drawn by plotting the individual LC_{50} values of the drugs in their respective X- and Y-axis. The LC_{50} values were obtained from the individual drugs effect of MTT assays. Then, a line was used to join both the data points and the LC_{50} value of the combined drug was spotted on the same plot. In principle, if the spotted point (LC_{50} value of combined drugs) falls on the line then it considered as additive, whereas if it falls below or above the line, then it considered as a synergistic or antagonist drug effect, respectively.

RESULTS

Cytotoxic effect of 5-FU, BCNU and Res in HCT-116 colon cancer cells

To determine the LC_{50} (concentrations needed for fifty percent cell death in culture) and to understand the cytotoxic effect of each drug on HCT-116 cells, the MTT assay was carried out. Three known chemotherapeutic agents (5-FU, BCNU and Res) were chosen for experimentation (Figure 1A). The cells were treated for 48 h before the MTT assay. Figure 1A shows that increasing the concentrations of each drug increased cell death. 5-FU was the most effective among the three drugs and BCNU was the least effective. LC_{50} was noted at 10 μmol/L 5-FU while 35 μmol/L Res and 60 μmol/L BCNU were needed to achieve the same amount of cell death (Figure 1A). Although 5-FU offered maximum cytotoxic effects on colon cancer cells, more than 50% of cells exhibited resistance[44]. To overcome such resistance and to increase the efficacy of Res, a combined treatment was employed comprising BCNU and Res. The BCNU concentration was kept constant at 20 μmol/L and varied concentrations of Res were added. The combination of BCNU with Res increased the sensitivity of HCT-116 cells. Fifty percent cell death was observed when 18 μmol/L Res was combined with 20 μmol/L BCNU (Figure 1B) ($P < 0.05$).

Detection of apoptosis in HCT-116 cells by DAPI staining

5-FU, BCNU and Res killed colon cancer cells, but to confirm whether the killing effect of the drugs was through apoptosis or necrosis, DAPI nuclear staining was performed. Figure 1C shows that the number of apoptotic nuclei increased with increasing concentrations of Res when combined with 20 μmol/L BCNU as compared with untreated cells. More than fifty percent apoptotic nuclei were observed when 20 μmol/L BCNU was associated with 15 μmol/L of Res. Figure 1D shows a graphical representation of the number of apoptotic and non-apoptotic nuclei ($P < 0.05$).

Combined effect of BCNU and Res caused DNA damage in HCT-116 cells

To determine whether a combination of BCNU and Res caused apoptosis through DNA damage or by another mechanism, we measured the DNA damaging efficiency of this combination in HCT-116 cells by DNA damage

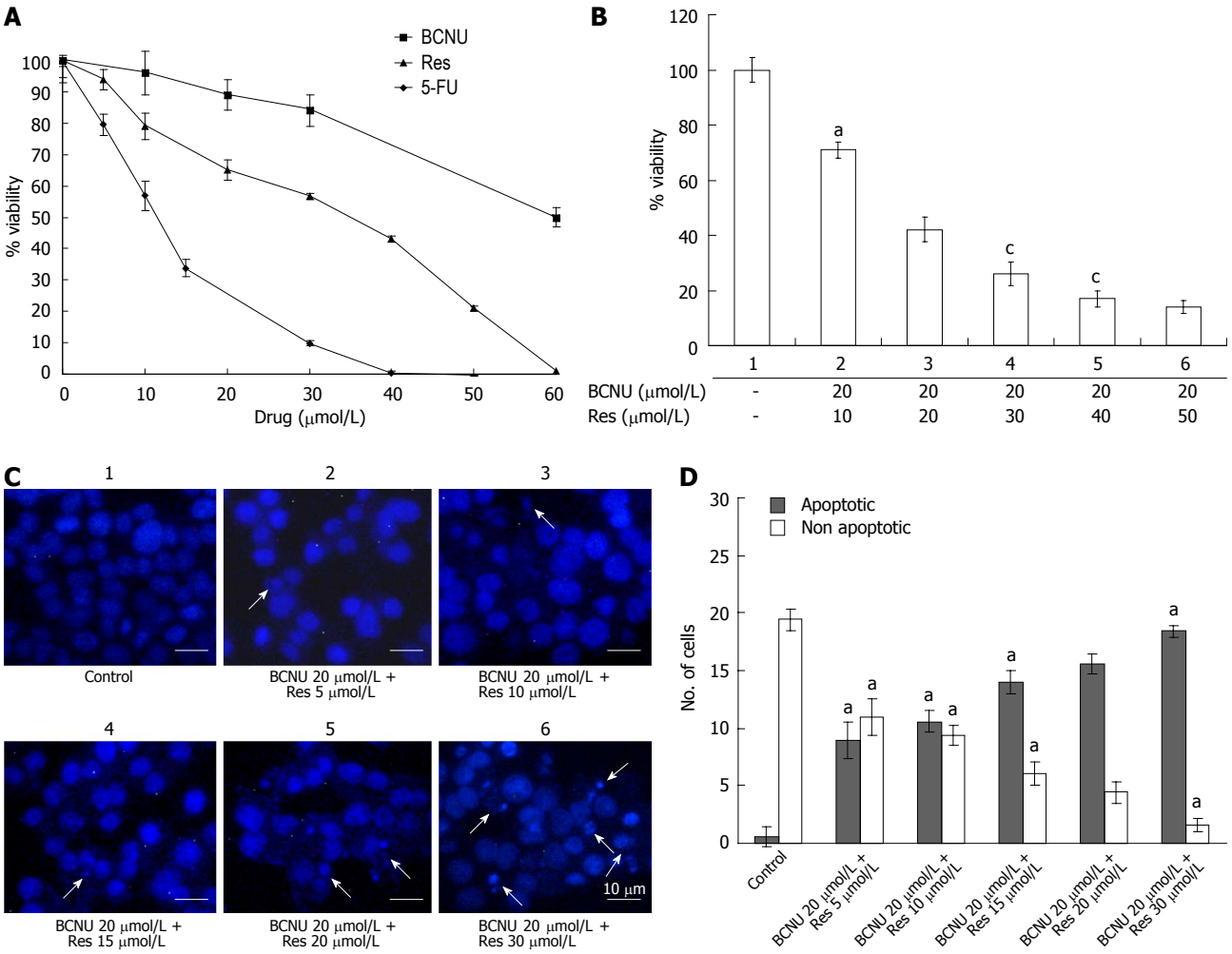


Figure 1 Anti-proliferative and apoptotic effect of 1,3-Bis(2-chloroethyl)-1-nitrosourea and/or resveratrol on HCT-116 colon cancer cells. A: Anchorage-dependent cell survival of HCT-116 cells after treatment with Res, 5-FU and BCNU; B: Bar diagram representing the % viability of HCT-116 after BCNU+ Res exposure. HCT-116 cells were cultured in 96 well plates and grown to 60%-70% confluence. The cells were then treated with different compounds according to the materials and methods. Data are the mean ± SD of three different experiments. $^{a}P < 0.05$ *vs* 20 μmol/L BCNU + 20 μmol/L Res; $^{c}P < 0.05$ *vs* 20 μmol/L BCNU + 50 μmol/L Res; C: Apoptotic nuclei after 4',6'-diamidino-2-phenylindole hydrochloride staining. Images were taken using a fluorescent microscope (Nikon-Eclipse, Japan) at × 40 magnification. Arrows indicate the apoptotic nuclei. Data are the representation of one of the replicates of three different experiments; D: A graphical representation of apoptotic nuclei. $^{a}P < 0.05$ *vs* 20 μmol/L BCNU + 20 μmol/L Res. 5-FU: 5-fluorouracil; Res: Resveratrol; BCNU: 1,3-Bis(2-chloroethyl)-1-nitrosourea.

assays (single cell gel electrophoresis or comet assay). The cells were pre-treated with 20 μmol/L BCNU for 24 h prior to exposure with various concentrations of Res for another 48 h. The comet formation and average comet length increased with increasing concentrations of Res (Figure 2A). Figure 2B shows the average comet length of combined treatment. Compared with untreated cells, the average comet length increased when Res was combined with BCNU (Figure 2B) ($P < 0.05$). Thus, the data suggests that the DNA damaging effect of Res was magnified in presence of BCNU.

Effects of BCNU and Res on the cell cycle regulation and apoptosis of HCT-116

It was reported that Res halts the cell cycle at S phase in various cancer cells, such as breast, colon and pancreas[45-49]. Similarly, it was also reported that BCNU arrests the cell cycle in the G2/M phase transition[50]. To determine the regulation of cell cycle profile by BCNU + Res combination, we treated the cells with the above-mentioned drugs and performed FACS analysis at the end of the exposure. The percentages of G_2/M population of cells decreased with increasing concentrations of Res combined with BCNU in comparison with the control (Figure 2C). The percentages of apoptotic cells (sub G_1) population increased in a dose dependent manner with Res combined with a fixed concentration of BCNU. Interestingly, approximately 60% apoptosis was noted when 30 μmol/L Res was combined with 20 μmol/L of BCNU (Figure 2C).

Effects of BCNU and Res on the expression level of apoptotic markers in HCT-116 cells

The combined effect of BCNU and Res on apoptosis, cell cycle regulation and DNA damage repair proteins in HCT-116 cells was studied by measuring the protein expression levels of well known markers, such as Bcl-2-associated X protein (BAX), B-cell lymphoma-extra large

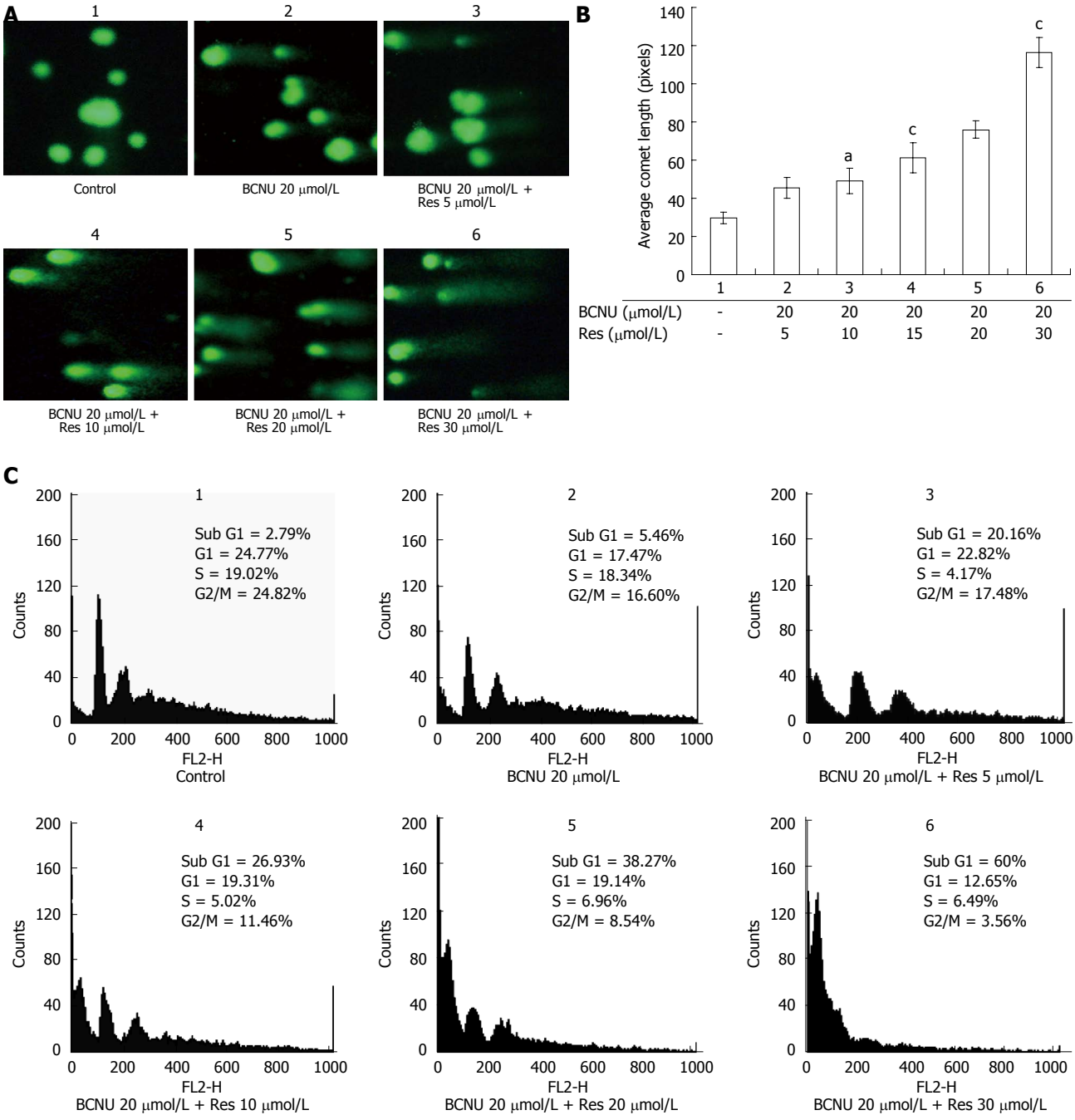


Figure 2 Regulation of cell cycle and genotoxicity of HCT-116 cells after 1,3-Bis(2-chloroethyl)-1-nitrosourea and/or resveratrol treatments. A: Comet assay showing the DNA damaging effect of the drugs in HCT-116 cells. Images were taken using a fluorescent microscope (Nikon-Eclipse, Japan) at × 20 magnification. Data are the representation of one of the replicates of three different experiments; B: Bar diagram represents the average comet - length in pixels as obtained from TriTek CometScore™ software. Data are the mean ± SD of three different experiments, $^{a}P < 0.05$ *vs* 20 μmol/L BCNU + 5 μmol/L Res; $^{c}P < 0.05$ *vs* 20 μmol/L BCNU + 20 μmol/L Res; C: Effect of the drugs on cell cycle regulation. After treatment as mentioned in the materials and method fluorescence-activated cell sorting analysis was performed and the DNA content of the cell was measured by Cell Quest Software (Becton and Dickinson, CA). Data are the representation of one of the replicates of three different experiments. 5-FU: 5-fluorouracil; Res: Resveratrol; BCNU: 1,3-Bis(2-chloroethyl)-1-nitrosourea.

(BCL-XL), Poly (ADP-ribose) polymerase (PARP), p21, proliferating cell nuclear antigen (PCNA), APE, phosphatase and tensin homolog (PTEN), FEN1 and human homolog of cyclin dependent kinase-2 (CDC-2) (Figure 3). The level of BAX increased compared with treatment with BCNU alone, whereas the level of BCL-XL decreased (Figure 3). The levels of CASPASE-3 and cleaved product of PARP also increased compared with BCNU alone (Figure 3). Thus, the data indicated that treatment with the BCNU and Res combination results in apoptosis in HCT-116 cells, as reflected by the elevated BAX/BCL-XL ratio, PARP cleavage and CASPASE-3 expression (Figure 3).

Effects of BCNU and Res on the expression level of cell cycle regulatory and DNA repair proteins in HCT-116 cells

DNA damage repair proteins, such as FEN1, APE, and

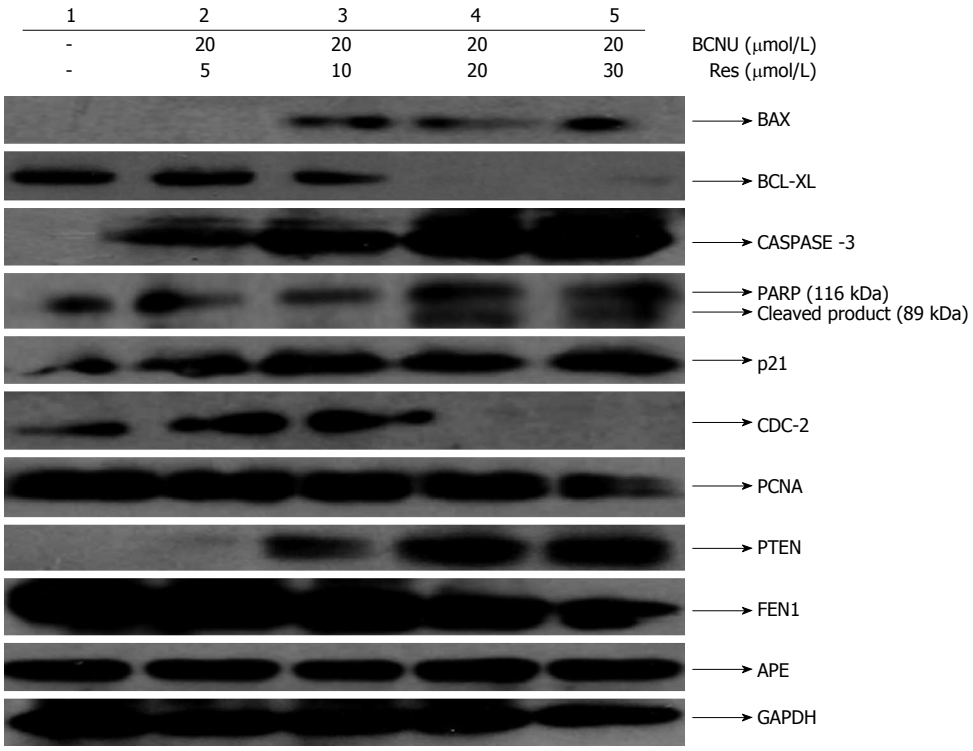


Figure 3 Combined effects of 1,3-Bis(2-chloroethyl)-1-nitrosourea and resveratrol on various cellular markers in HCT-116 colon cancer cells. Expression pattern of apoptotic, DNA damage/repair and cell cycle regulatory proteins after drug treatment. Data are the representation of one of the replicates of three different experiments. Glyceraldehyde phosphate dehydrogenase (GAPDH) served as a loading control. BAX: Bcl-2-associated X protein; BCL-XL: B-cell lymphoma-extra large; PARP: Poly (ADP-ribose) polymerase; PCNA: Proliferating cell nuclear antigen; PTEN: Phosphatase and tensin homolog; FEN1: Flap endonuclease 1; CDC-2: Cyclin dependent kinase-1; Res: Resveratrol; BCNU: 1,3-Bis(2-chloroethyl)-1-nitrosourea; APE: Apurinic/apyrimidinic (AP) endonuclease.

PCNA, were assayed to determine the effects on DNA damage/repair in HCT-116 cells after BCNU and Res combination treatment (Figure 3). FEN1 and PCNA levels decreased when the dose of Res increased, whereas the level of APE remained almost constant during combination treatment (Figure 3). The cell cycle regulatory protein p21 and PTEN increased, while CDC-2 decreased, after combined treatment for 48 h. PTEN is considered to be a cell cycle regulatory tumor suppressor protein, and an increase in PTEN expression indicates the anti-tumor property of the combined drug. Increased p21 expression during the combination treatment indicated negative cell cycle regulation. A decrease in CDC-2 expression also indicated negative regulation of the cell cycle. Thus, these experiments demonstrated that treatment with a combination of BCNU and Res induced apoptosis in colon cancer cells by affecting cell cycle regulation and DNA damage/repair. Therefore, this novel drug combination can be used for treating 5-FU resistant cells.

Establishment of a 5-FU-resistant stable cell line

The 5-FU-R cell line was further characterized by measuring the expression pattern of anti-apoptotic, cell cycle and DNA damage repair proteins, such as nuclear factor kappa-light-chain-enhancer of nuclear factor κB (NFκB), FLICE-like inhibitory protein (FLIP), casein kinase 1 (CK1), disabled homolog 2 (DAB2), glycogen synthase kinase 3 beta (GSK-3β), AKT, POLH, TS, POL-β, DDB2, and FEN1, which were altered in stable cell line compared to normal HCT-116 cells (Figure 4). The expression levels of AKT, NFκB, FLIP, POLH, GSK-3β, POL-β, DDB2, and FEN1 were increased in comparison with HCT-116 cells. However, the levels of CK1 and DAB2 expressions decreased compared with HCT-116 cells (Figure 4). Interestingly, widely established 5-FU resistance protein thymidylate synthase also increased in 5-FU-R cells compared with HCT-116 cells (Figure 4).

Cytotoxicity of the BCNU and Res combination in 5-FU-R cells

The experiments discussed above indicated that BCNU and Res combination significantly increased the sensitivity of HCT-116 colon cancer cells. To determine the optimum concentration of the BCNU and Res combination for cytotoxicity of 5-FU-R cells, an MTT assay was carried out. BCNU showed similar cytotoxic profile in 5-FU-R cells compared with HCT-116 cells (LC_{50} 55 μmol/L) (Figure 5A); however, it was noted that 5-FU resistant cells were more sensitive to Res compared with HCT-116 parental cells. The LC_{50} of Res in 5-FU-R cells was 18 μmol/L, but was 35 μmol/L in HCT-116 cells (Figure1A *vs* Figure 5A). Interestingly, it was noted that there was little or no effect of 5-FU on 5-FU-R cells, even after treatment with 100 μmol/L for 48 h (Figure 5A). This data suggests that 5-FU-R cells are resistant to 5-FU, but sensitive to other chemotherapeutic drug, such

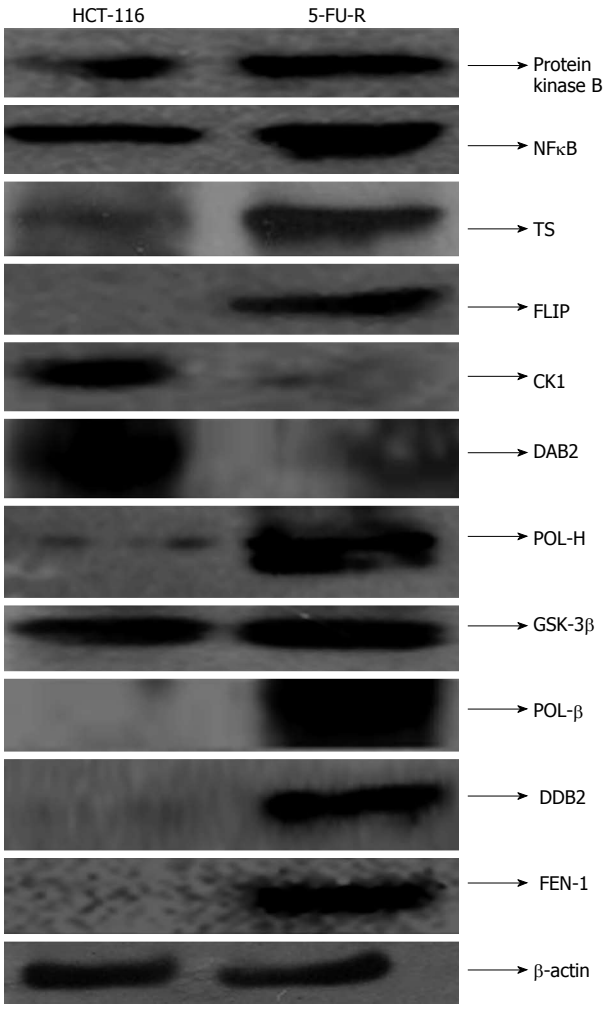


Figure 4 Characterization of 5-fluorouracil-resistant cells. The cell lysates of HCT-116 and 5-FU-R cells were immunoblotted using specific antibody. The lower panel shows the expression of β-actin, which was the loading control (to ensure the same amount of protein loaded in each lane). Data are the representation of one of the replicates of three different experiments. CK1: Casein kinase 1; FLIP: FLICE-like inhibitory protein; NFκB: nuclear factor κB; POL-β: DNA polymerase beta; POLH: DNA polymerase eta; protein Flap FEN1: Endonuclease 1; DDB2: DNA damage-binding protein 2; 5-FU: 5-fluorouracil; Res: Resveratrol; BCNU: 1,3-Bis(2-chloroethyl)-1-nitrosourea; TS: Thymidylate synthase.

as Res.

A dose dependent decrease in cell viability was observed when increasing concentrations of Res were added to the 20 μmol/L BCNU pre-treated 5-FU-R cells (Figure 5B). Fifty percent cell death (LC_{50}) was noticed when 8 μmol/L of Res was combined with 20 μmol/L of BCNU (Figure 5B) ($P < 0.05$). Interestingly, it was noted that lower concentrations of Res were needed to cause fifty percent cell death in 5-FU-R cells compared with HCT-116 cells (LC_{50} for HCT-116 cells was 18 μmol/L *vs* LC_{50} for 5-FU-R cells was 8 μmol/L). Thus, it appeared that 5-FU-R resistant cells (Figure 5B) were more susceptible than HCT-116 cells (Figure 2B) to the BCNU and Res combination.

Apoptosis of 5-FU-R cells was measured by DAPI nuclear staining after exposure to the optimum formulation (BCNU 20 μmol/L + Res 8 μmol/L), BCNU and Res. Although treatment with BCNU and Res alone caused cell death, increased cell death was observed by treatment with the combination compared with BCNU and Res alone (Figure 5C). However, Res alone caused more apoptosis than BCNU alone (Figure 5C). Figure 5D shows the graphical representation of the number of apoptotic and non-apoptotic nuclei ($P < 0.05$).

Combined effect of BCNU and Res caused increased DNA damage, and decreased cell cycle regulatory protein expression and apoptosis in 5-FU-R cells

To elucidate the underlying mechanism of the sensitivity 5-FU-R cells to the BCNU and Res combination, a series of experiments were carried out. To determine whether the BCNU and Res combination caused DNA damage, a comet assay was performed after treatment with BCNU 20 μmol/L + Res 8 μmol/L for 48 h. The average comet length and number of comets increased with the combination treatment compared with BCNU and Res alone or untreated cells (Figure 6A). Figure 6B shows the average comet length, in pixels, of 5-FU-R cells treated with BCNU and Res alone and in combination [BCNU (20 μmol/L) +Res (8 μmol/L)] ($P < 0.05$). The average comet length almost doubled when 8 μmol/L of Res was combined with BCNU compared with BCNU alone. The percentage of apoptosis and cell cycle profile cells were also measured by FACS analysis after treatment with the combined drugs (Figure 6C). Increased accumulation of the G_2/M (24%) population was noted after the BCNU treatment in comparison to Res (19.33%) and untreated cells (10%). Interestingly, the increase in G_2/M population decreased when Res was combined with BCNU; however, the observed percentage of apoptosis (sub G_1) was as high as 70%. To further confirm the apoptosis and to determine the status of various protein biomarkers, Western blotting was carried out. Figure 6D shows the expression patterns of apoptotic markers, such as cleaved product of PARP, BAX, CASPASE-3 and BCL-XL. The cleaved product of PARP and the BAX/BCL-XL ratio increased with increasing concentration of the combined drug. Cell cycle regulatory proteins, such as p21 and PTEN increased, while CDC-2 decreased after combined treatment for 48 h, indicating that this drug combination has adverse effect on cell cycle regulation in the 5-FU-R cells. Figure 6E represents the expression pattern of DNA repair proteins when treated with the combined drug and Res and BCNU alone. The levels of FEN1, POLH, DDB2 and POL-β were decreased in cells that were treated with BCNU and Res alone compared with the control, but the combination treatment showed a radical decrease in the levels of these proteins compare with the control and treatment with each drug alone (Figure 6E). Interestingly, the level of tumor suppressor and DNA repair protein APC was completely abolished after individual (BCNU and Res) treatment, but increased after combined drug exposure compared with untreated cells. This result clearly indicates that the BCNU and Res works additively or synergistically and their mode of ac-

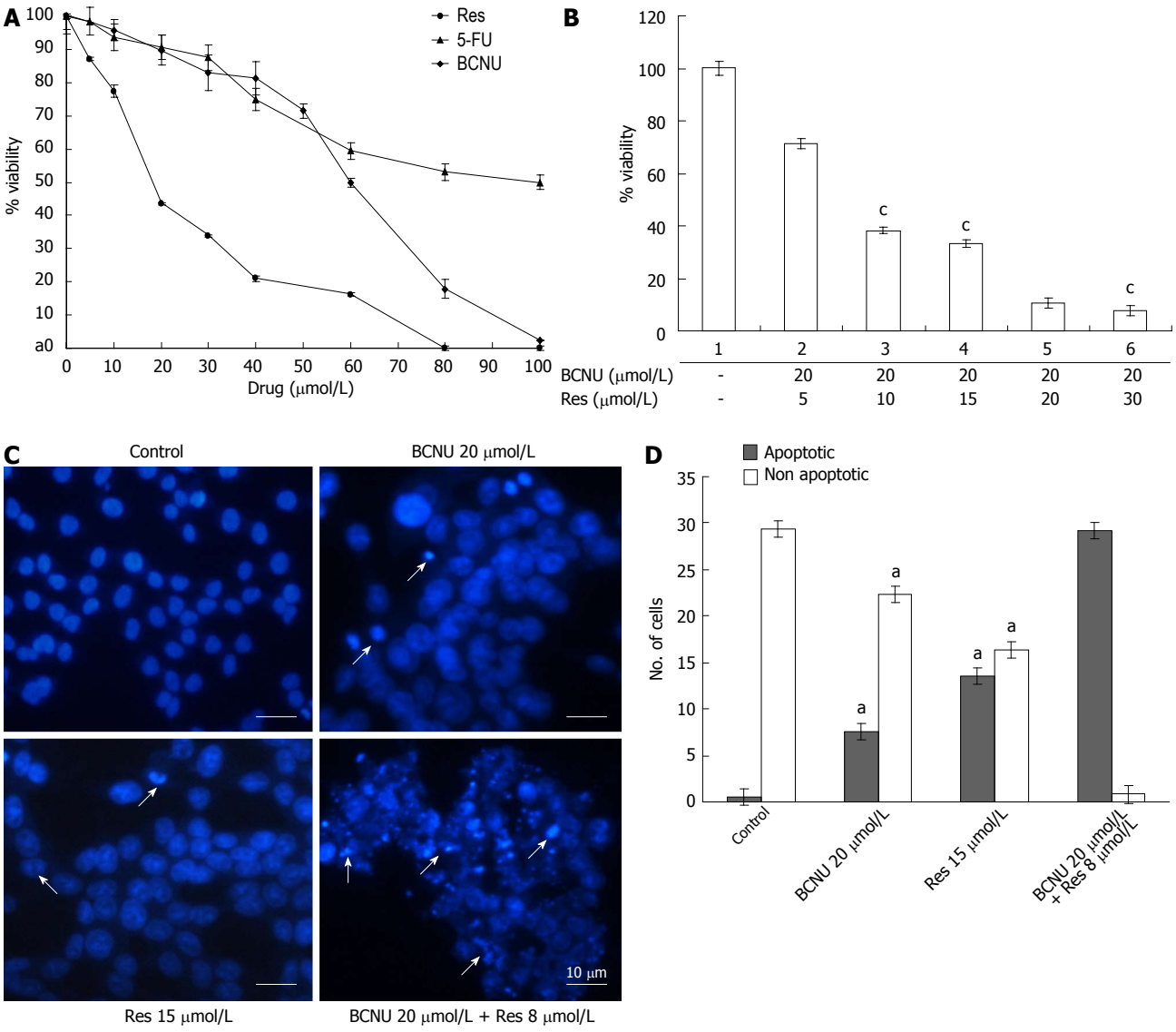


Figure 5 Anti-proliferative and apoptotic effect of 3-Bis(2-chloroethyl)-1-nitrosourea and/or resveratrol on 5-fluorouracil-R cells. A: Anchorage-dependent cell survival of 5-FU-R cells after treatment with 5-FU, Res and BCNU; B: Bar diagram representing the % viability of 5-FU-R cells after BCNU + Res exposure. Data are the mean ± SD of three different experiments, $^{a}P < 0.05$ *vs* 20 μmol/L BCNU + 5 μmol/L Res; $^{c}P < 0.05$ *vs* 20 μmol/L BCNU + 20 μmol/L Res; C: Apoptotic nuclei after DAPI staining. Images were taken using a fluorescent microscope (Nikon-Eclipse, Japan) at ×40 magnification. An arrow indicates the apoptotic nuclei. Data are the representation of one of the replicates of three different experiments; D: A graphical representation of apoptotic nuclei, $^{a}P < 0.05$ *vs* 20 μmol/L BCNU + 8 μmol/L Res. 5-FU: 5-fluorouracil; Res: Resveratrol; BCNU: 1,3-Bis(2-chloroethyl)-1-nitrosourea.

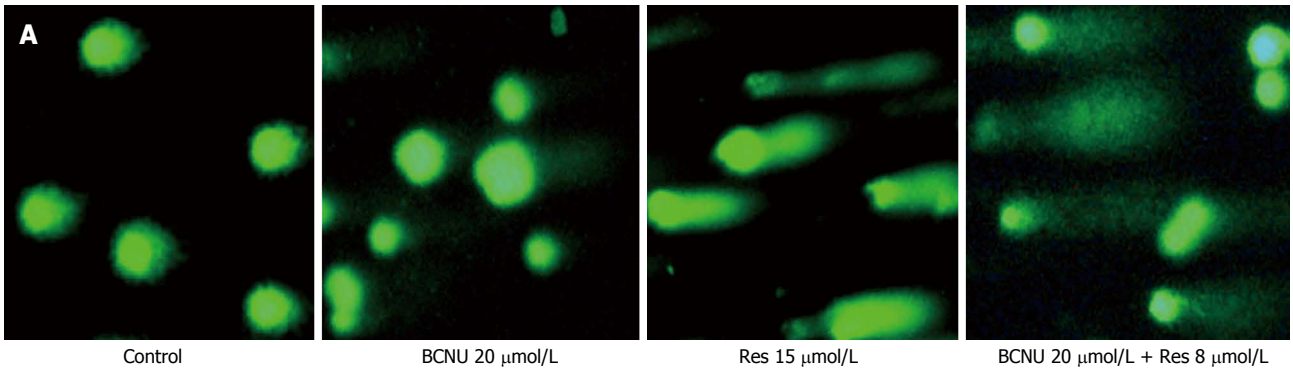
tion was to modulate DNA damage/repair pathway.

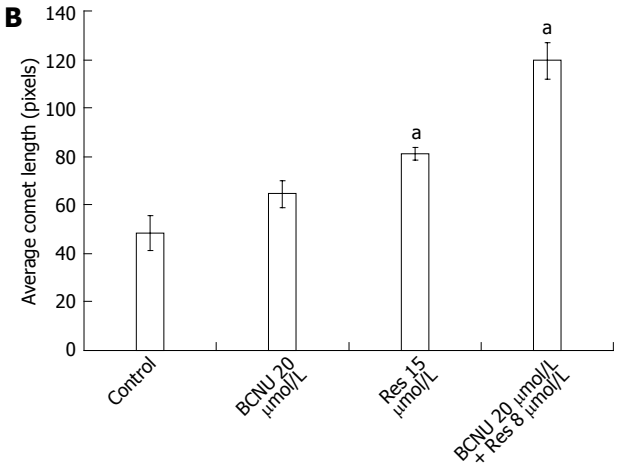
BCNU and Res synergistically increase the sensitivity in human colon cancer cells

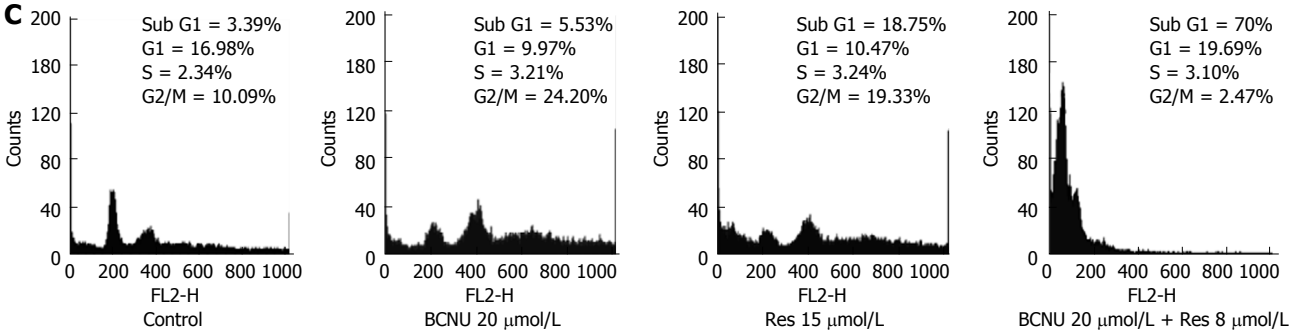
The above data showed that the BCNU and Res combination increased the sensitivity of colon cancer cells. To determine whether these two drug work additively or synergistically, we performed isobologram analysis. Figure 6F shows the occurrence of a synergistic interaction of Res and BCNU in both the HCT-116 and 5-FU-R cell lines. The LC_{50} value of the Res and BCNU in HCT-116 cells were 30 and 60 μmol/L, respectively, but for the combination, fifty percent cell death occurred at 20 μmol/L BCNU and 18 μmol/L Res (Figure 2B), which were much lower than dose of each individual drug (point Q). Point Q appeared below the lines, which suggested a synergistic interaction of these drugs. To further investigate the synergistic interaction of these drugs against the 5-FU-R cells, the same isobologram was plotted using the LC_{50} value of MTT data from Figure 5B. Interestingly, the fifty percent cell death (point P) with the combined drug appeared below the line, clearly indicating synergistic activity of BCNU and Res in 5-FU-R cells (Figure 6F).

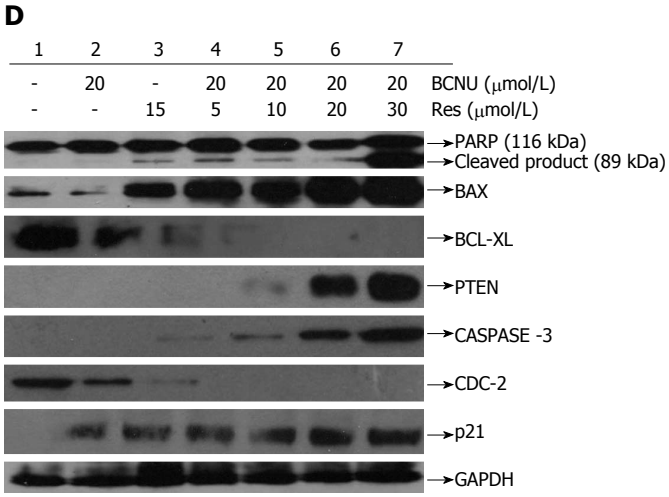
DISCUSSION

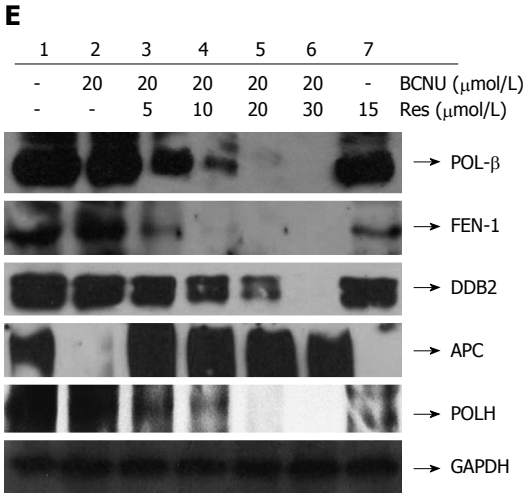
5-FU is widely used for the treatment of many types of cancers, including colorectal, breast, and cancers of the aerodigestive tract. Notably, 5-FU is routinely employed in the management of colorectal cancer *via* one of the two FDA-approved first line combinatorial chemothera-


A
Control
BCNU 20 μmol/L
Res 15 μmol/L
BCNU 20 μmol/L + Res 8 μmol/L


B
Average comet length (pixels)
0
20
40
60
80
100
120
140
a
a
Control
BCNU 20 μmol/L
Res 15 μmol/L
BCNU 20 μmol/L + Res 8 μmol/L


C
Counts
Sub G1 = 3.39%
G1 = 16.98%
S = 2.34%
G2/M = 10.09%
Control
Sub G1 = 5.53%
G1 = 9.97%
S = 3.21%
G2/M = 24.20%
BCNU 20 μmol/L
Sub G1 = 18.75%
G1 = 10.47%
S = 3.24%
G2/M = 19.33%
Res 15 μmol/L
Sub G1 = 70%
G1 = 19.69%
S = 3.10%
G2/M = 2.47%
BCNU 20 μmol/L + Res 8 μmol/L
FL2-H


D
1 2 3 4 5 6 7
- 20 - 20 20 20 20 BCNU (μmol/L)
- - 15 5 10 20 30 Res (μmol/L)
PARP (116 kDa)
Cleaved product (89 kDa)
BAX
BCL-XL
PTEN
CASPASE -3
CDC-2
p21
GAPDH


E
1 2 3 4 5 6 7
- 20 20 20 20 20 - BCNU (μmol/L)
- - 5 10 20 30 15 Res (μmol/L)
POL-β
FEN-1
DDB2
APC
POLH
GAPDH

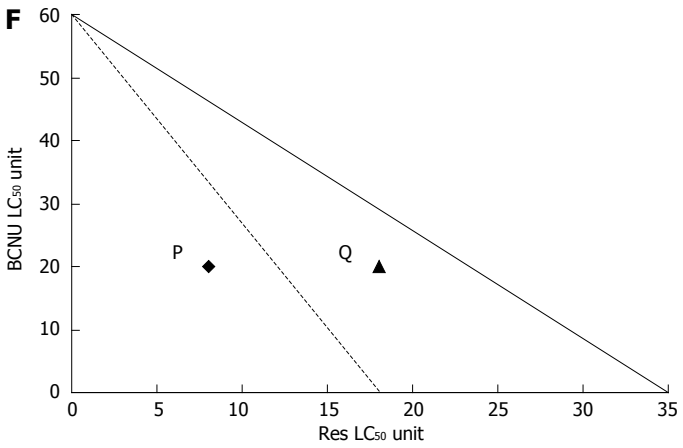


Figure 6 1,3-Bis(2-chloroethyl)-1-nitrosourea and Res synergistically increased apoptosis in 5-fluorouracil-resistance cells. A: Comet assay. Images were taken using a fluorescent microscope (Nikon-Eclipse, Japan) at × 20 magnification. Data are the representation of one of the replicates of three different experiments; B: Bar diagram representing the average comet score of the treated and untreated 5-FU-R cells in pixels, as obtained from TriTek CometScore™ software. Data are the mean ± SD of three different experiments, $^{a}P < 0.05$ *vs* 20 μmol/L BCNU; C: Regulation of cell cycle and apoptosis by BCNU, Res and their combination in 5-FU-R cells. Fluorescence-activated cell sorting analysis was done after the desired treatment and then the DNA content was measured by Cell Quest Software. Data are the representation of one of the replicates of three different experiments; D, E: Western blotting of 5-FU-R cell lysate after treatment with BCNU and Res individually, as well as in combination. Glyceraldehyde-3-phosphate dehydrogenase was used as the loading control. Data are the representation of one of the replicates of three different experiments; F: Isobolograms for combinations of BCNU and Res in HCT-116 and 5-FU-R cells. The bold line represents the isobologram of BCNU and Res in HCT-116 and dotted lines represents the isobologram of BCNU and Res in 5-FU-R cells. The point P and Q represent the 50% cell death of the combined drug in case of 5-FU-R and HCT-116 cells, respectively. 5-FU: 5-fluorouracil; Res: Resveratrol; BCNU: 1,3-Bis(2-chloroethyl)-1-nitrosourea.

py regimes, which involve intravenous administration of the fluorinated base analog[10,11]. One problem with 5-FU treatment is that more than 50% of tumors demonstrate resistance to5-FU in the clinical setting. Thus, novel treatments are needed to treat these resistant tumors. Novel biological agents, such as the monoclonal antibodies Cetuximab (an epidermal growth factor receptor inhibitor) and Bevacizumab (a vascular endothelial growth factor inhibitor), have recently been shown to provide additional clinical benefit for patients with metastatic colorectal cancer. However, these agents have demonstrated marginal results when treating resistant tumors[51,52].

During the past few decades, investigators have attempted to elucidate the mechanism underlying 5-FU resistance[10-12]. Some investigators developed 5-FU resistant cell lines and studied their sensitivity to multiple chemotherapeutic agents by activation and inhibition of several signaling pathways[53,54]. Unfortunately, none of these agents showed promising results.

In the present report, we developed a new strategy for overcoming 5-FU resistance by establishing 5-FU-R cell lines and studying their sensitivity to a combination treatment with BCNU and Res. BCNU and Res display anticancer properties by inducing DNA damage, arresting cell cycle progression, inhibiting anti-apoptotic makers and autophagy[13,28]. We have shown recently that moderately low concentration of 5-FU (0.5 μmol/L) increased chemoprevention potential of Res in colon cancer cells[7]. However, in that report we could not eliminate the probability of 5-FU resistance because of presence of 5-FU. Thus, that cocktail will not be appropriate for the treatment of 5-FU resistance patients. In the present study, we treated the cells with BCNU and Res to increase the sensitivity of 5-FU resistant cell lines. Initially, we checked whether BCNU and Res combination increase the sensitivity of 5-FU sensitive HCT-116 cells. LC_{50} was observed at 60 μmol/L BCNU and 35 μmol/L Res; however, the same amount of cell death was observed when 18 μmol/L Res was combined with 20 μmol/L BCNU. Reduction of both BCNU and Res concentration for fifty percent cell death revealed that BCNU and Res killed HCT-116 colon cancer cells synergistically (Figure 1B). The isobologram data (Figure 6F) also support the synergistic action of BCNU and Res to kill colon cancer cells.

Cell death associated with the synergistic effects of treating cells with a combination of BCNU and Res was further confirmed by multiple parameters, such as DAPI nuclear staining, comet assay, FACS analysis and measuring the expression patterns of several DNA damage/repair, cell cycle regulatory and apoptosis related protein biomarkers. Increased numbers of apoptotic nuclei after DAPI stain, average comet formation and comet length, percentage of sub G_1 population were observed after combined treatment (BCNU + Res) in HCT-116 cells. An increased ratio of BAX/BCL-XL, cleaved product of PARP, tumor suppressor PTEN, cell cycle regulatory protein p21, and CASPASE-3 were also noted in BCNU and Res combination. Thus, the data suggests that the BCNU and Res combination represents a potential treatment option for 5-FU resistant colon cancer.

We have developed a 5-FU resistant cell line from 5-FU sensitive HCT-116 cells by continuous treatment with 5-FU. These cells were resistant to 5-FU, but sensitive to other chemotherapeutic agents, such as Res. The cell death effects of the BCNU and Res combination in the resistant cell lines were measured. MTT cell survival, DAPI nuclear staining and comet assays showed that the

BCNU + Res combination was highly effective to sensitize the 5-FU-R cells in comparison to individual compounds. They caused cell death synergistically. The FACS analysis showed approximately 70% cell death when 20 μmol/L BCNU combined with 8 μmol/L Res (Figure 6C). By contrast, approximately 60% cell death was observed when 20 μmol/L of BCNU combined with 30 μmol/L Res in HCT-116 sensitive cells. Increased percentages (70% *vs* 60%) of apoptosis even at lower concentration of Res (8 μmol/L *vs* 30 μmol/L) indicated that Res was more effective to sensitize the 5-FU-R than HCT-116 cells when combined with BCNU. The expression of PARP cleaved product and BAX/BCL-XL ratio increased with the combined treatment (BCNU + Res) in 5-FU-R cells compared with Res and BCNU alone (Figure 6D). Isobologram analysis also indicated that BCNU and Res also killed the 5-FU-R cells synergistically.

To study the mechanism of action of the BCNU and Res combination, we have first analyzed whether the DNA damage/repair pathway was involved. Comet assays showed that the average comet length and comet formation increased in both 5-FU sensitive and resistant cell lines after treatment with the BCNU + Res combination. Interestingly, a much lower concentration of Res (8 μmol/L) was needed to cause the same comet length in 5-FU-R cells compared with 5-FU sensitive cells (Res 30 μmol/L) (Figure 2B *vs* Figure 6B). This observation suggests that Res was more effective in causing DNA damage in 5-FU-R cells than in 5-FU sensitive cells when associated with the same amount of BCNU. This data prompted us to check the level of DNA damage/repair related proteins after treatment with BCNU + Res in 5-FU-R cells. Interestingly, the major DNA repair proteins, such as FEN1, POLH, DDB2 and POL-β decreased in case of combination treatment while the level of APC increased in comparison to the individual drug as well as untreated cells (Figure 6E).

Multiple pieces of evidence indicate that the repair of apurinic/apyrimidinic (AP)-sites in DNA occurs through two sub-pathways of BER, which differ on the basis of repair gap size and the enzymes involved in these repair pathways[55]. These sub-pathways are designated as "single-nucleotide BER" or "short patch (SP)-BER" and "multinucleotide BER" or "long patch (LP)-BER". In both pathways, repair is started by removal of a damaged base by a DNA glycosylase leaving an abasic (AP-site) DNA. The resulting AP sites are subsequently acted upon by an APE to generate a 3' hydroxyl group and a 5'-deoxyribosephosphate (dRP) terminus. In SP-BER, Pol-β extends the 3' terminus by a single nucleotide and removes the dRP moiety with its dRP lyase activity. Finally, the nick is sealed by DNA ligases[56]. However, the oxidized or reduced AP sites become resistant to β-elimination and cannot be excised by the dRP lyase activity of Pol-β. Then the modified AP-site is repaired *via* the LP-BER pathway, in which Pol-β, d or ε incorporates 2-15 nucleotides, displacing the strand containing the modified AP-site. Then, the DNA flap structure is cleaved by FEN1, and the nick is sealed by a DNA ligase[57,58]. APC expression is induced in cancer cell lines upon exposure to DNA-damaging agents[39,40,59,60], suggesting an interaction between APC and the DNA repair machinery. We have already shown that APC physically interacts with Pol-β, FEN1 and blocks BER by blocking strand-displacement synthesis and then DNA repair[39-42]. Increased levels of APC and decreased levels of Pol-β and FEN1 after combined treatment with BCNU + Res revealed that this cocktail kills cancer cells by inhibiting the BER pathway. Thus, the above result indicates that BCNU + Res in combination caused cell death through reduction of BER repair pathway. The possible cause of this effect might be activation of APC by the combination but not the individual treatments.

A new and emerging concept is to sensitize cancer cells to DNA-damaging agents by inhibiting various proteins in DNA repair pathways. Molecular docking or NMR studies identified small molecular weight inhibitors (SMIs) that target the BER pathway by inhibiting APE1 and Pol-β activities[61]. Although a number of Pol-β inhibitors have been reported in recent years[61,62], more potent and selective inhibitors are still needed. Since abasic DNA damage (which may also be caused by BCNU) can also be repaired by LP-BER, there is a need for agents that can block the LP-BER pathway as well, in which 5'-flap endonuclease 1 (FEN1) plays a major role[63]. FEN1 recognizes and removes the 5'-flap structure generated by Pol-β during the strand-displacement synthesis. The removal of this flap is essential for the joining of the newly synthesized DNA strand with the parent strand by DNA ligase to complete the repair. Interestingly, the FEN1 level decreased after BCNU and Res combination in both cell lines, indicating that the BCNU + Res combination inhibited the removal of flap from the newly synthesized step in LP-BER. BCNU + Res might cause more DNA damage that could not be repaired by the cells because of a lack of sufficient FEN1. This would result in mutations accumulating within the cells, eventually leading to apoptosis instead of survival. However, more study will be needed to understand the molecular mechanism involving the BER pathway triggered by the BCNU + Res combination.

In conclusion, this study showed that a combination of BCNU and Res could be useful to overcome 5-FU resistance in colon cancer patients. BCNU and Res caused apoptosis in both the HCT-116 cells and 5-FU-R cells by inhibiting the base excision repair, especially *via* the FEN1 protein of the LP-BER pathway. These findings represent a novel concept for overcoming 5-FU resistance in colon cancer patients.

COMMENTS

Background

Colon cancer is one of the most common causes of cancer-related death worldwide. 5-fluorouracil (5-FU) is a widely used chemotherapeutic drug for the treatment of colon cancer, but resistance to this drug is a barrier to successful chemotherapy.

Research frontiers

1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) is a synthetic drug commonly used to treat brain tumors as it can pass through the blood-brain barrier. BCNU is highly effective against cancer, but causes toxicity at very high concentrations. Resveratrol is a plant-derived compound used to treat various forms of cancer. Res has low toxicity and efficacy. Combining BCNU with resveratrol (Res) increases the efficacy of Res and reduces the toxicity of BCNU, making the combination an good tool to inhibit HCT-116 cells and overcome 5-FU resistance. Moreover, these drugs are DNA damage specific, resembling similar mechanism of action to that of 5-FU by reducing DNA repair components. Hence, these drugs were chosen for the combination therapy.

Innovations and breakthroughs

This report highlighted the importance of combination therapy, which surpassed the monotherapy involving BCNU and Res to inhibit the growth of HCT-116 cells and overcome 5-FU resistance. This is the first study to report a combination of BCNU and Res for the treatment of colon cancer and to overcome 5-FU resistance effectively. The authors reported the mechanism of action of the combined drug, *i.e.*, inhibition of the DNA damage/repair pathway and arresting the cell cycle, thereby leading to apoptosis. The authors also reported that the drug to be more effective in the resistant cells than the normal cancer cells.

Applications

This study highlights the action of BCNU combined with Res, which could prove to be a major tool for the treatment of 5-FU sensitive/resistant colon cancer patients and could also be tested for its ability to overcome resistance to other drugs, as this cocktail had specificity towards drug resistant cells.

Terminology

BCNU is a common synthetic alkylating chemotherapeutic agent belonging to the family of haloethylnitrosoureas. Resveratrol is a polyphenolic phytoalexin widely available in plants, such as red grapes.

Peer review

The research work in this manuscript is about the tumor suppression action of BCNU in combination with resveratrol on 5-fluorouracil HCT-116 cell lines, which were made resistant to 5-FU on constant exposure of 5-FU. The study is interesting and well executed. The point is clearly made.

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ISSN 1007-9327
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