

Inhibition of the p38 MAPK pathway sensitises human colon cancer cells to 5-fluorouracil treatment

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Abstract. Colorectal cancer is the third most common cause of cancer-related deaths in the Western world. 5-Fluorouracil (5-FU) based chemotherapeutic regimes have been the mainstay of systemic treatment for disseminated colorectal cancer for many years. However, it only produces a 25% response rate due to the drug-resistance. The mitogen-activated protein kinase (MAPK) pathway is involved in the anti-apoptotic process; its activation provides cancer cells with a survival advantage to escape the apoptotic challenge. This study assessed whether the p38 MAPK pathway is involved in 5-FU resistance in colorectal cancer cells. 5-FU only or 5-FU combined with a p38 MAPK pathway inhibitor (SB203580) was used to treat 5-FU-resistant colorectal cancer cells. The effect of the treatment on cell viability, death and caspase activities was assessed. Western blotting was used to investigate the responses of apoptosis-related proteins following the treatment. Results showed that p38 MAPK inhibitor significantly increased colorectal cancer cell sensitivity to 5-FU. SB203580 in combination with 5-FU significantly reduced cell viability ($P<0.01$), and increased cell death and cellular caspase activity ($P<0.01$). Western blotting data revealed that SB203580 sensitises cancer cells to 5-FU due to an increase in Bax expression. These findings suggest that p38 MAPK is involved in cancer cell survival, and that the inhibition of p38 MAPK can enhance 5-FU to kill colorectal cancer cells.

Introduction

Colorectal cancer (CRC) is the third most common cause of cancer-related deaths among men and women in the Western

world (1,2). Europe has the highest CRC incidence with an estimated 412,900 new cases in 2006 (1) closely followed by the USA which had 145,290 colorectal cancers in 2008 (3). 5-Fluorouracil (5-FU)-based chemotherapeutic regimes have been the mainstay of systemic treatments in CRC for many years. However 5-FU treatment is not very effective against disseminated CRC, overall it only produces 10-20% response rate (4). One major reason for 5-FU failure to treat disseminated CRC is the development of drug resistance (5). Tumours usually consist of mixed populations of malignant cells, some of which are drug-sensitive while others are drug-resistant. Chemotherapy kills drug-sensitive cells but leaves behind a higher proportion of drug-resistant cells, which can expand and maintain tumour growth in spite of repeated treatment. One way to overcome drug-resistance is to sensitise the cancer cells to 5-FU treatment.

5-FU is an anti-tumour drug widely used in the treatment of solid tumours (6). Its anti-tumour mechanism has been associated with inhibition of thymidylate synthase (TS) and induction of genotoxic stress by incorporation of 5-FU into RNA and DNA (6). Resistance to 5-FU is a multi-factorial event which includes altered drug-transport mechanisms, metabolic conversion, molecular mechanisms, protection from apoptosis and resistance via cell cycle kinetics (7). Because of the reactivity of 5-FU and complexity of the cellular response to DNA and RNA damage, 5-FU-induced apoptotic signalling is likely to involve several pathways. Elucidation of the details of these pathways will help us to understand why some tumour cells exposed to 5-FU often lose sensitivity and become resistant to apoptotic signals.

Chemotherapy-reduced genotoxic stress often induces multiple signal transduction pathways including mitogen-activation protein kinase (MAPK). MAPK has been associated with anti-apoptotic processes and chemo-resistance development in CRC cells (8). MAPK has been found to be activated aberrantly in CRC patients (9). We hypothesised that MAPK activation may provide cancer cells with a survival advantage, and assist malignant cells escaping from the drug-induced apoptotic challenge. To test this, we treated 5-FU-resistant CRC cells with 5-FU supplemented with or without MAPK pathway inhibitors, and assessed the effects of the treatment on cell viability, cell death and caspase activity. It was found that inhibition of p38 MAPK pathway increased colorectal

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cancer cell (HT29) sensitivity to 5-FU treatment. Further studies showed that the increased sensitivity to 5-FU treatment was mediated with the elevation of a pro-apoptotic protein-Bax.

Material and methods

Reagents. Caspase 3/7, 8 and 9 assay kits and cell viability assay kits were purchased from Promega. Yo Pro-1 iodide was purchased from Invitrogen. p38 MAPK and MEK1/2 MAPK inhibitors were purchased from Merck Bioscience. Antibodies against human p38 and phospho-p38, p42/44 and phosphor-p42/44 were purchased from New England Bio-labs. Anti-human Bax and Bcl2 antibodies were purchased from Santa Cruz Biotechnology. 5-Fluorouracil (50 mg/ml) was manufactured in Medac (Germany) and purchased from the Royal Free Hospital pharmacy department. All cell culture media, sera and antibiotics were purchased from Gibco.

Cell lines and culture condition. The human colon cancer cell lines (HT29, HCT116) were purchased from European Collection of Cell Cultures (ECACC). All cells were cultured and maintained in DMEM medium containing 10% foetal bovine serum and 1% penicillin and streptomycin at 37°C with 5% CO₂/95% air. Cells used in all experiments were at 3-8 passages after their receipt from the supplier.

Establishment of 5-FU resistance in colorectal cancer cells. HT29 and HCT116 human colorectal cancer cell lines were used to assess 5-FU resistance. Cells were seeded in 96-well plates with DMEM medium containing 10% FBS and 1% PenStrep at the density of 2×10^4 cells/well in 37°C with 5% CO₂/95% air for 24 h. After seeding, cells were washed three times with medium and then treated with 5-FU for 48 h. Following the treatment, cells were subjected to cell death and viability assays. In order to verify that 5-FU resistance exists in these cancer cells, both cell lines were subjected to the treatment with a wider range of concentration of 5-FU for 48 h. All experiments were performed in triplicate and repeated on two separately-initiated cultures (n=6).

Determination of which MAPK pathways affected by 5-FU treatment. HT29 CRC cells were seeded in 6-well plates with DMEM medium containing 10% FBS and 1% PenStrep at the density of 1.8×10^5 cells/well in 37°C with 5% CO₂/95% air for 24 h. Following the seeding, cells were treated by 5-FU for 48 h. The level of p38, phospho-p38, p42/44 and phosphor-p42/44 was determined by Western blotting. The experiments were performed in duplicate and repeated on two separately-initiated cultures. The value of protein level in each treatment was expressed as the mean of 4 samples (n=4).

Inhibition of p38 MAPK pathway. After establishment of 5-FU resistance, only 5-FU resistant cells (HT29) were used in the following experiments. Cells were seeded on 96-well plates with DMEM medium containing 10% FBS and 1% PenStrep at the density of 2×10^4 cells/well in 37°C with 5% CO₂/95% air for 24 h. After seeding, cells were treated with or without 5-FU in combination with a p38 MAPK inhibitor (SB203580) or a MEK1/2 MAPK inhibitor (MEK1/2 inhibitor)

for 48 h. Following the treatment, cells were subjected to cell viability, death and caspase activity assays. The experiments were performed in triplicate and repeated on two separately-initiated cultures (n=6).

Determination of the level of anti- and pro-apoptotic proteins following 5-FU treatment combined with p38 MAPK inhibition. HT29 CRC cells were seeded in 6-well plates with DMEM medium containing 10% FBS and 1% PenStrep at the density of 1.8×10^5 cells/well at 37°C with 5% CO₂/95% air for 24 h. Following the seeding, cells were divided into 4 groups as follows, i) treated with 5-FU (5 µg/ml), ii) received 5-FU (5 µg/ml) and SB203580 (5 µM) treatment, iii) treated with 5-FU (5 µg/ml) and SB203580 (10 µM), and iv) was untreated as the control group. The levels of Bax and Bcl2 were determined by Western blotting. The experiments were performed in duplicate and repeated on two separately-initiated cultures. The value of protein level in each treatment was expressed as the average of samples (n=4).

Cell viability assay. Cell viability was assessed using a previously reported method (10). For comparison, all cell viability data were presented as the percentage of treated cells to untreated cells, which was calculated using the following formula: cell viability (%) = $F_t/F_c \times 100$; where F_t represents fluorescence reading for treated cells and F_c represent fluorescence reading for untreated cells in the cell viability assay.

Cell death assay. Cell death assessment was carried out with a previously described method (10,11). For comparison, the cell death index for treated and untreated cells was calculated with the formula: cell death index = F_t/F_c ; where F_t and F_c represent the units of fluorescence (RLU) in the treated (F_t) and the untreated (F_c) cells, respectively.

Caspase activity assay. Caspase 8 and 9 activity was assessed with Caspase-Glo assay kit using a previously reported method (12). For comparison, all caspase activities were presented as increase fold-change compared to untreated cells.

Protein extraction and Western blotting. Cells were washed 3 times with PBS, lysed by the addition of RIPA buffer containing protease inhibitor cocktail (Roche) and harvested with a cell scraper. In order to assist cell lysis, cell suspensions were repeatedly frozen and thawed. SDS-PAGE was used to separate the proteins. The primary antibody dilution for Bax, Bcl2 was 1/200, for p38, phospho-p38, p42/44 and phosphor-p42/44 was 1/1000. The density of protein bands was measured using densitometry software (Molecular Analyst, Bio-Rad image analysis software V1.5, USA).

Statistical analysis. All data have been examined and demonstrated a normal distribution. All results were expressed as mean ± SEM. One way ANOVA (Prism version 4 2004 edition, USA) with multiple comparison tests were used. Statistical analysis was performed on samples (n=4-6) and Bonferroni's multiple comparison test was used. $P < 0.05$ was considered statistically significant, $P < 0.01$ highly significant. In figures, indicated as * $P < 0.05$ and ** $P < 0.01$, respectively.

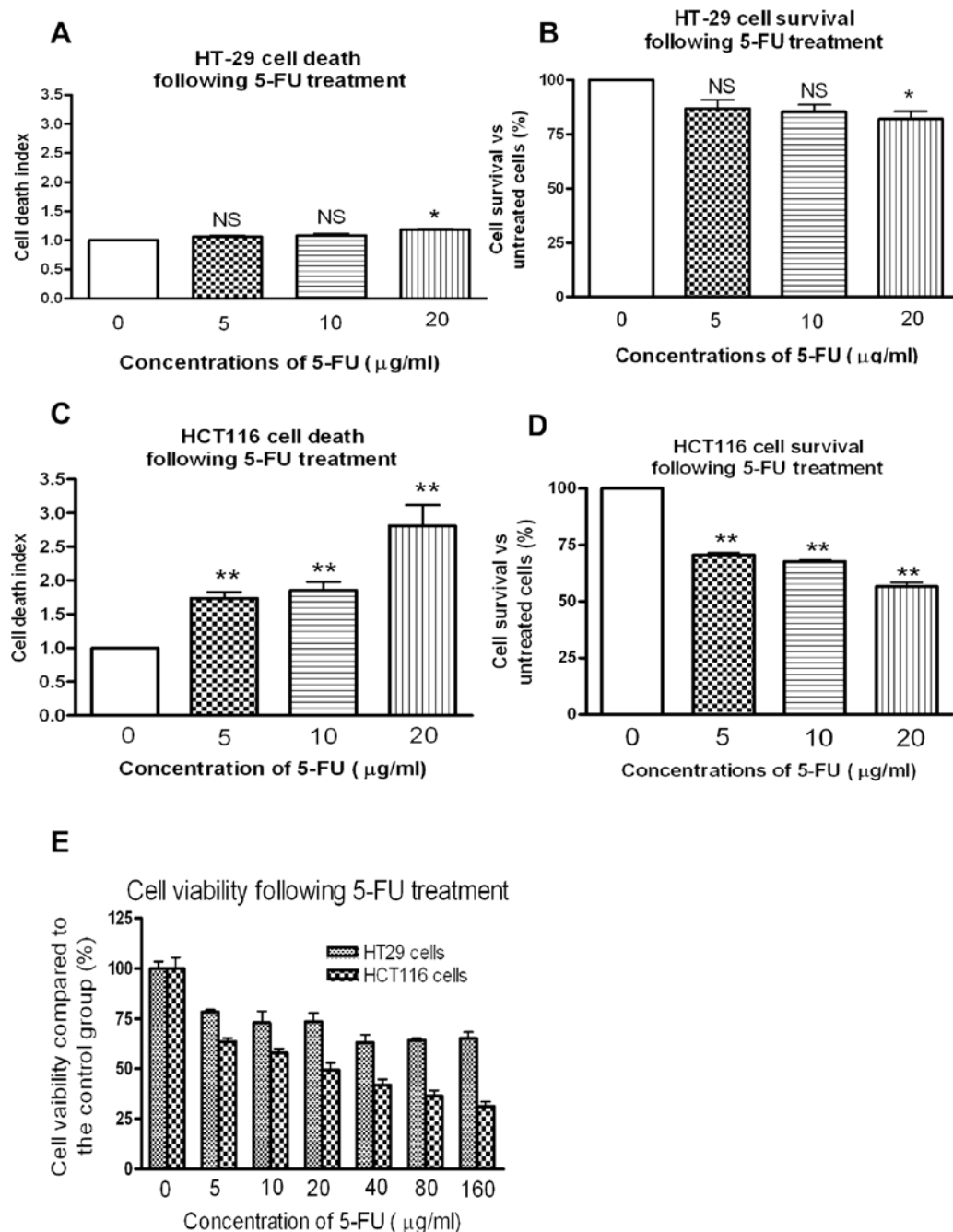


Figure 1. Human colon cancer HT29 (A and B) and HCT116 (C and D) cells were treated with 5-FU. Cell viabilities (B and D) and death index (A and C) were determined. The results are shown as mean \pm SEM, (n=6). The viability of two cell lines were assessed following expose to a wide range of concentrations of 5-FU for 48 h, the results (E) showed that when 5-FU concentration increased, the viability of HCT116 cell continuously decreased. The viability of HT29 cell only responded to the first few concentrations, but failed to change in response to the higher doses of 5-FU.

Results

Establishment 5-FU resistance in colorectal cancer cells.

Two cell lines (HT29 and HCT116) were used to assess the resistance to 5-FU in CRC cells. Although 5-FU treatment was shown to induce cell death and reduce cell viability in both cell lines in a dose-dependent manner (Fig. 1), the sensitivity to the treatment was different in the two cell lines. HCT116 cells were more sensitive to the treatment. All doses of 5-FU induced significantly higher levels of cell death, and markedly reduced cell viabilities in HCT 116 cells (Fig. 1C and D). HT29 cells were much less sensitive to the treatment;

only higher doses of 5-FU (20 $\mu\text{g/ml}$) induced higher levels of cell death and reduced cell viabilities significantly. Lower doses of 5-FU (5 and 10 $\mu\text{g/ml}$) failed to kill HT29 cells (Fig. 1A and B). To further verify that 5-FU resistance exists in HT29 cells, two cell lines were treated with a wide range of concentrations of 5-FU, the result was shown in Fig. 1E. When 5-FU concentration increased, the viability of HCT116 cell continuously decreased. However the viability of HT29 cells only responded to the first few concentrations, but failed to response to the higher doses of 5-FU (Fig. 1E). This result established that HT29 cells are 5-FU resistant which also was confirmed by a recent study (13). Therefore, only the HT29

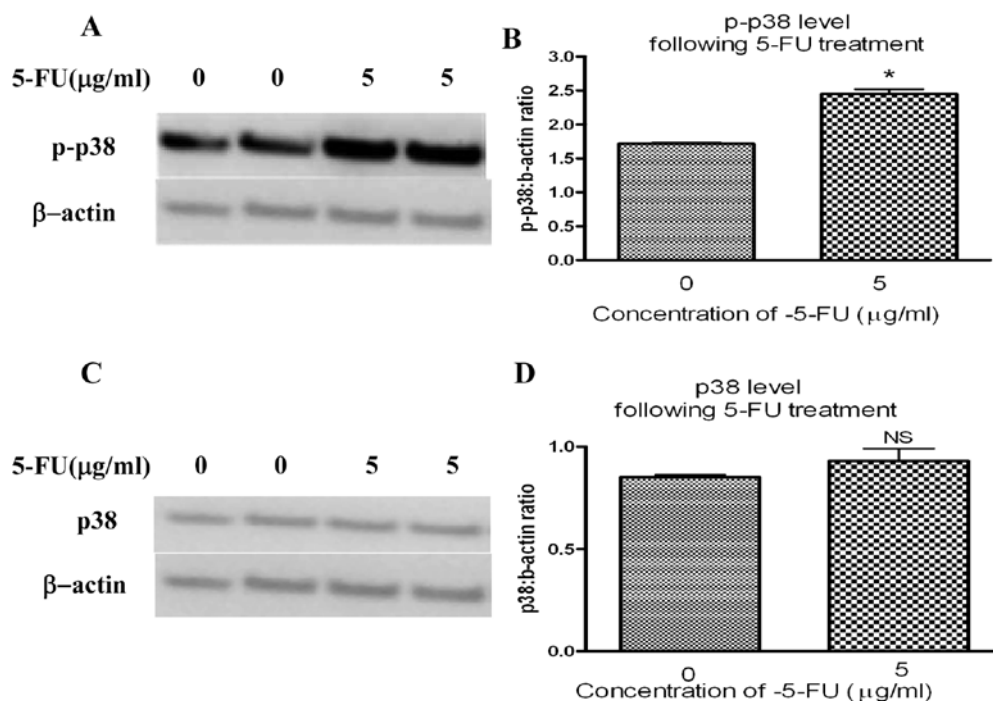


Figure 2. HT29 CRC cells were treated by 5-FU for 48 h. Total protein was isolated from cells and separated with SDS-PAGE electrophoresis. The bands of phospho-p38 (A) and p38 (C) were identified by polyclonal antibodies against human phospho-p38 and p38, respectively. The level of phospho-p38 (B) and p38 (D) was determined by measurement of protein band using densitometry software. The results are shown as mean \pm SEM, (n=4).

cells were used in the following investigation to sensitise cancer cells to 5-FU.

Determination of which MAPK pathway was affected by 5-FU treatment. The responses of two major signalling cascades (p38 and MEK1/2 MAPK pathways) to 5-FU were investigated. Results showed that the p38 MAPK pathway was affected by the treatment (Fig. 2). Following 48 h of 5-FU treatment, the levels of p38 protein were not significantly changed (Fig. 2C and D, $P>0.05$), but the level of phospho-p38 was elevated significantly compared to the untreated cells (Fig. 2A and B, $P<0.05$). This suggested that 5-FU significantly increased p38 phosphorylation in HT29 cells. For the MEK MAPK cascade, it was found that both levels of p42/44 and phospho-p42/44 were not changed following the treatment, indicating that MEK MAPK pathway was not affected by 5-FU actions (Fig. 3).

Inhibition of p38 MAPK enhances 5-FU-induced cell death and reduces cell survival in CRC cells. In order to assess whether inhibition of p38 MAPK affected 5-FU treatment on HT29 cells, a p38 MAPK inhibitor (SB203580) was combined with 5-FU to treat HT29 cells. When cells were treated by 5-FU alone, there was no significant difference in cell death (Fig. 4A) and viability (Fig. 4B) compared to the untreated control cells, but if 5-FU was supplemented with SB203580, cell death significantly increased (Fig. 4A). Similar effects were also observed in cell viability assays (Fig. 4B). It is interesting to note that SB203580 enhanced 5-FU-induced cell death (Fig. 4A) and reduced cell viability (Fig. 4B) in a dose-dependent manner. All these data suggested that the inhibition of p38 MAPK pathway can sensitise HT29 cells to 5-FU.

Inhibition of MEK1/2 MAPK does not affect HT29 cancer cell sensitivity to 5-FU treatment. To investigate the role of MEK1/2 MAPK pathway in the development of 5-FU resistance in CRC cells, a MEK1/2 MAPK inhibitor (MEK1/2 inhibitor) was combined with 5-FU to treat HT29 cells. The results (Fig. 4C and D) showed that inhibition of MEK1/2 pathway did not affect the outcome of 5-FU treatment. There were no differences in cell death in any of the treated cells, including those treated by 5-FU alone and 5-FU supplemented with MEK1/2 inhibitor compared to untreated cells (Fig. 4C). This indicated that MEK1/2 MAPK is not involved with cell apoptosis induced by 5-FU. For cell viability, 5-FU treatment alone decreased the viable cell slightly, but the addition of MEK1/2 inhibitor failed to enhance 5-FU-reduced cell viability (Fig. 4D). All these data suggested that MEK1/2 MAPK pathway had no effect on 5-FU treatment in CRC cells, which is consistent with some previous studies (14,15).

Inhibition of p38 MAPK significantly increases caspase 8 and 9 activity. Activation of caspases has been regarded as a hallmark for cell apoptosis. Further investigation was carried out to assess the effect of inhibition of p38 MAPK on the activity of caspases 8 and 9. Results show that the SB203580 combination with 5-FU was able to increase the activity of both caspase 8 and 9 significantly compared to untreated and 5-FU only treated cells (Fig. 5A and B). The SB203580 alone is also able to activate both caspases, but the combination appears to have more potential to stimulate both enzymes activities. Caspase 8 activation is one part of extrinsic apoptotic cascades and caspase 9 activation is one part of intrinsic apoptosis pathway. SB203580 treatment activated both caspase 8 and 9 indicating that interfering with p38 MAPK pathway induced CRC cell extrinsic and intrinsic apoptotic death.

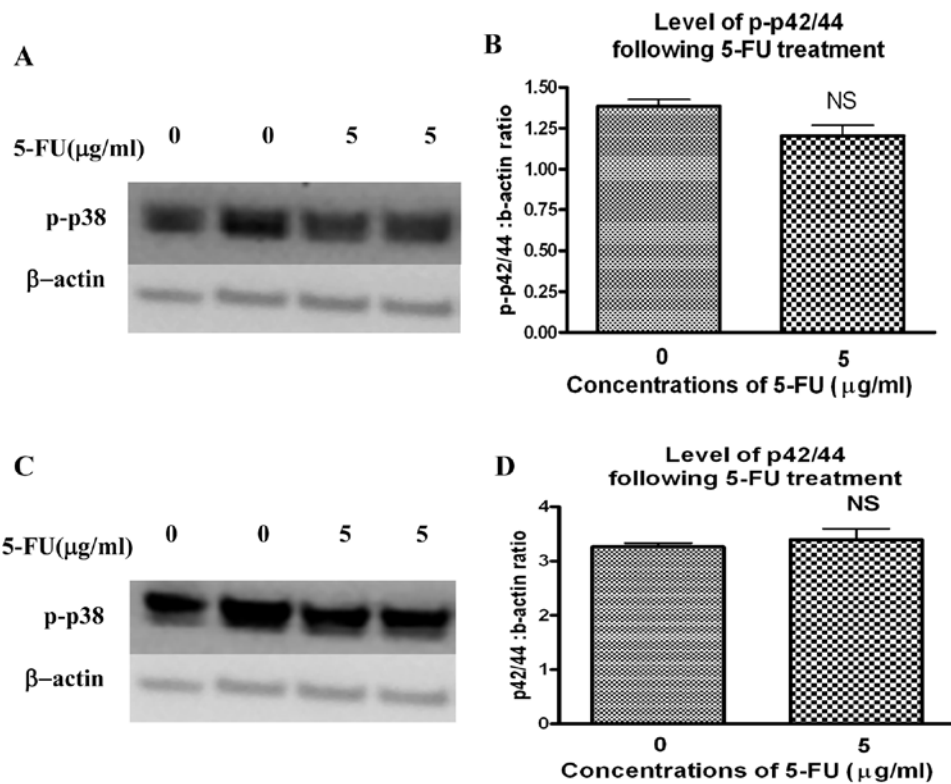


Figure 3. HT29 CRC cells were treated with 5-FU. Total protein was isolated from cells and separated with SDS-PAGE electrophoresis. The bands of phospho-p42/44 (A) and p42/44 (C) were identified by polyclonal antibodies against human phospho-p42/44 and p42/44, respectively. The levels of phospho-p42/44 (B) and p42/44 (D) were determined by measurements of protein band density using densitometry software. The results are shown as mean \pm SEM, (n=4).

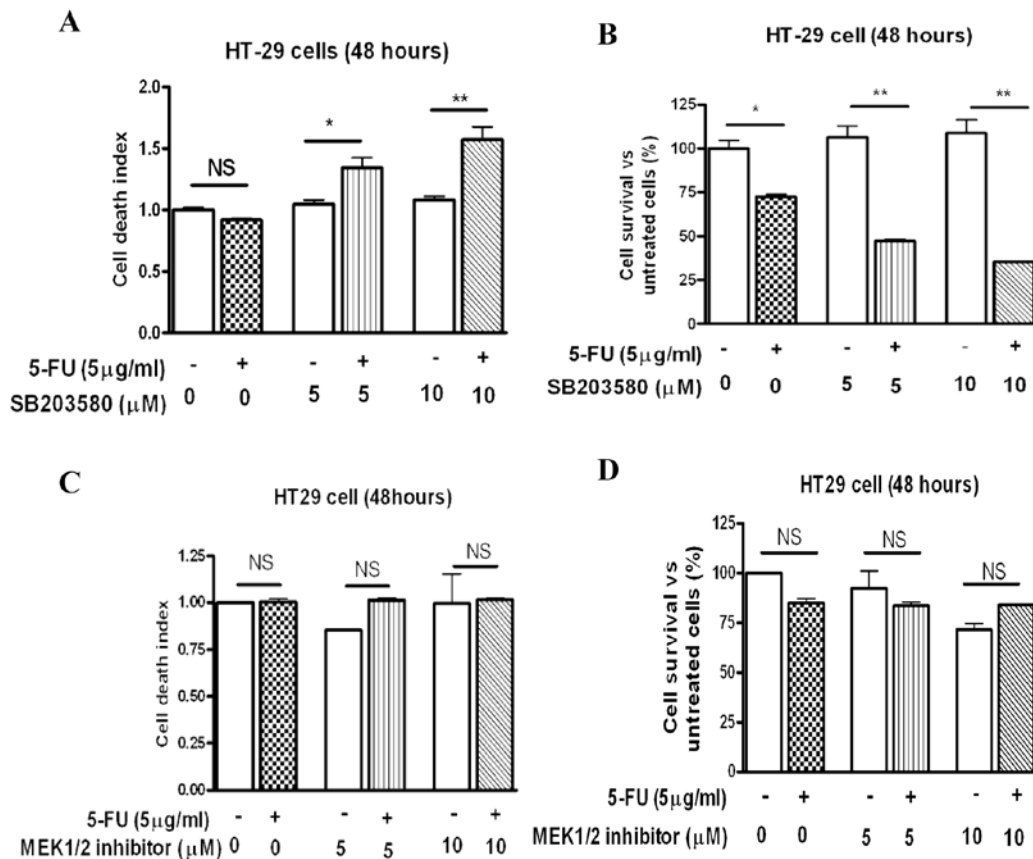


Figure 4. HT29 CRC cells were treated with 5-FU supplemented with or without SB203580 (A and B) or MEK1/2 inhibitor (C and D) inhibitor for 48 h. Cell death index (A and C) and viabilities (B and D) were determined by a previous reported method. The results are shown as mean \pm SEM, (n=6).

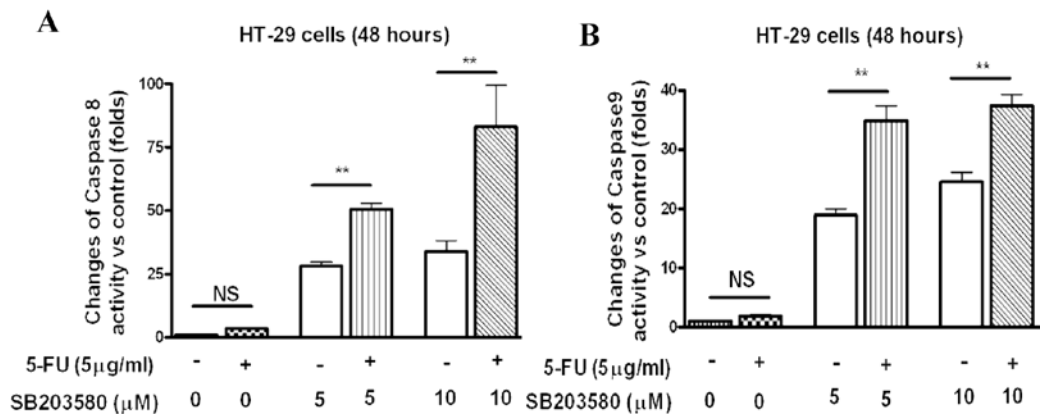


Figure 5. HT29 CRC cells were treated with 5-FU supplemented with or without SB203580 at concentrations of 5 and 10 µM for 48 h. The activities of caspase 8 (A) and 9 (B) were determined by Caspase-Glo assays. For comparisons, all caspase activities were presented as increase in fold compared with untreated cells. All results were expressed as means ± SEM, (n=6).

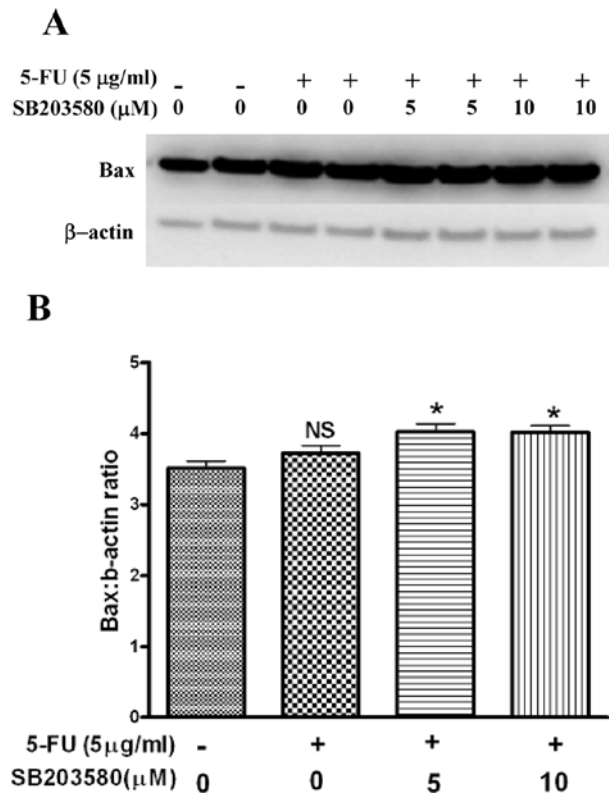


Figure 6. HT29 CRC cells were treated with 5-FU supplemented with SB203580. Total protein was isolated from cells and separated with SDS-PAGE electrophoresis. The bands of Bax (A) and β-actin were identified by polyclonal antibodies against human Bax and β-actin. The level of BAX and β-actin was determined by measuring the density of protein bands using densitometry software and expressed as Bax: β-actin ratio (B). Results are shown as mean ± SEM, (n=4).

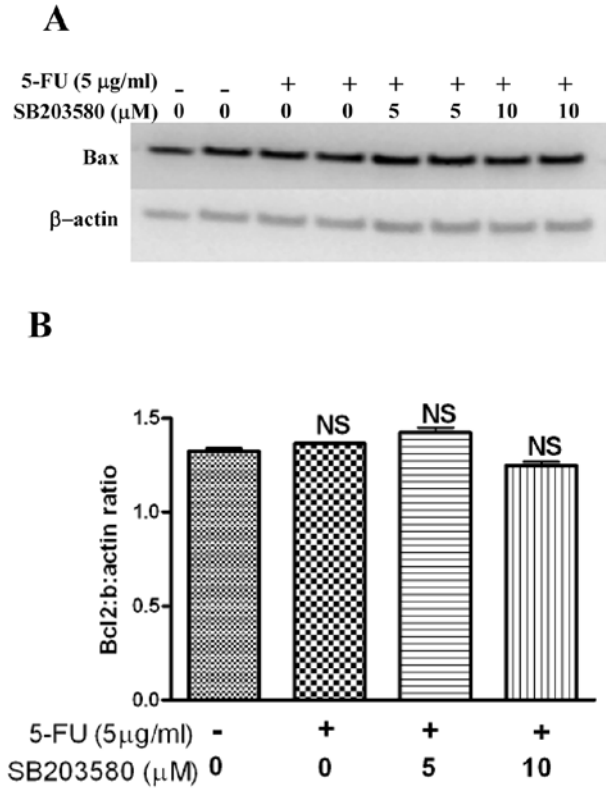


Figure 7. HT29 CRC cells were treated with 5-FU supplemented with SB203580. Total protein was isolated from cells and separated with SDS-PAGE electrophoresis. The bands of Bcl2 (A) and β-actin were identified by polyclonal antibodies against human Bcl2 and β-actin, respectively. The levels of Bcl2 and β-actin were determined by measuring the density of protein bands using densitometry software and expressed as Bcl2: β-actin ratio (B).

Inhibition of p38 MAPK increases pro-apoptotic protein Bax, but does not affect anti-apoptotic protein Bcl2. To test whether 5-FU supplement with p38MAPK have any effects on apoptotic regulation proteins, the expression levels of Bax (a pro-apoptotic protein) and Bcl2 (an anti-apoptotic protein) were examined by Western blotting following 5-FU alone or 5-FU supplemented with SB203580 treatment. The results

are shown in Figs. 6 and 7. It is shown that 5-FU alone was not able to alter expression of either Bax or Bcl2 (Figs. 6 and 7). When cells were treated with 5-FU supplemented with SB203580 (5-10 µM), the level of pro-apoptotic protein Bax significantly increased (Fig. 6A and B). However, such effects were not observed in the anti-apoptotic protein Bcl2. These data suggested that 5-FU treatment supplemented with

SB203580-induced HT29 cell death was via the increase in the expression of pro-apoptotic protein Bax.

Discussion

This study examined the effects of 5-FU on p38 MAPK and MEK1/2 pathways in a 5-FU-resistant CRC cell line, and found that 5-FU significantly increased p38 phosphorylation in CRC cells, which may give an advantage for cell survival from 5-FU treatment. When cancer cells were treated by 5-FU supplemented with p38 MAPK inhibitors, the advantage of survival disappeared and significant cell death was induced. The cell death appears to be correlated with increased expression of a pro-apoptotic protein, Bax. Further studies have confirmed that 5-FU supplemented with the inhibition of p38 MAPK significantly increased caspase 8 and 9 activity which is the hallmark of cellular apoptosis. We also found that 5-FU did not affect the MEK1/2 MAPK pathway, and inhibition of the MEK1/2 MAPK pathway did not enhance 5-FU killing in HT29 cells. All data together suggested that p38 MAPK is involved in cancer cell survival following 5-FU treatment, and the inhibition of P38 MAPK can enhance 5-FU killing CRC cells.

The mitogen-activation protein kinase (MAPK) pathway is a major signal transduction cascade which ultimately triggers multiple biological cell responses following growth factor treatment or stress stimulation (16). So far several sub-families of MAPKs including p38 MAPK pathway (17) and extracellular signal-regulated kinase (MEK1/2) pathway (18) have been identified. P38 MAPK pathway is primarily activated in response to environmental stress and mainly involved in cell growth arrest and apoptosis (17). It can be stimulated by various stresses such as UV and γ irradiation, osmotic stress, heat shock and some chemotherapeutic drugs (19). A recent study (20) found that inhibition of p38 MAPK down-regulated a multidrug-resistance I gene, which was believed to be responsible for chemotherapy resistance in gastric cancer cells. However, to date we could not find any report providing insight into the details of the role of p38 MAPK pathway in 5-FU resistance in CRC cells. In this study we examined the effects of 5-FU treatment on p38 MAPK pathways and found that 5-FU increased p38 phosphorylation. Inhibition of p38 MAPK markedly enhanced 5-FU-induced CRC cell death, and significantly improved 5-FU-reduced CRC cell viability and increased caspases 8 and 9 activities, which up-regulate apoptosis induced by 5-FU. The mechanism of p38 MAPK inhibition to sensitise cancer cells to the 5-FU may be due to the increases in the expression of a pro-apoptotic protein Bax.

The MEK1/2 pathway is mainly activated by mitogen and growth factors, and participates in cellular processes such as cell proliferation and differentiation (18). The role of MEK signalling in cancer cell chemoresistance remains contradictory. Some studies have found that activation of MEK pathway is associated with resistance to apoptosis-induced by chemotherapeutic drugs, and inhibition of the MEK signalling leads to increased sensitivity of cancer cells to chemotherapy (21-23), alternatively some studies have found that inhibitors of MEK1/2 MAPK blocked chemotherapy-induced apoptosis (24,25), whilst other studies have demonstrated that modulation of MEK1/2 MAPK does not affect chemoresistance

profiles in cancer cells (14,15). We found that the inhibition of the MEK1/2 MAPK pathway has no effect on 5-FU treatment in CRC cells, which is consistent with some of these contradictory findings (14,15). The role for MEK 1/2 MAPK pathway in determining cell survival or death is far from clear (23). The different effects observed suggest that the role of MEK1/2 pathway in chemoresistance is dependent on cell type and conditions.

Bax and Bcl-2 are two main proteins which mediate mitochondrial membrane permeability (26). Bax is a pro-apoptotic protein, which forms a trans-membrane pore across the outer mitochondrial membrane, leading to loss of membrane potential and enhanced apoptosis. Bcl-2 is an anti-apoptotic protein, which prevents the pore formation and cell death (27). The ratio of Bcl2 to Bax can be a marker to indicate the propensity of cells to undergo apoptosis (26). Our study shows that when cells were treated with 5-FU supplemented with an inhibitor of p38 MAPK the level of pro-apoptotic protein (Bax) significantly increased, but the level of anti-apoptotic protein (Bcl2) was not affected. This suggests that the inhibition of p38 MAPK induces Bax expression, which in turn, modulated the loss of mitochondrial membrane potential, finally contributed to cancer cell death.

A recent study (28) which analysed data from >20,000 CRC patients in 18 randomised trials has shown that 5-FU-based chemotherapy has improved overall survival in CRC patients, but it also identified that the early recurrence of the cancer was a great challenge to further improve the response rates. There are clear correlations between chemoresistance and cancer recurrence. 5-FU-induced cancer cell apoptosis involves many cellular signalling pathways. Investigation into the detail of these pathways will help us to understand why some tumour cells exposed to 5-FU often lose sensitivity. This study provides more details of the role of the p38 MAPK pathway in the development of 5-FU resistance in CRC cells, and could be used to optimise adjuvant agents to improve the response rate of chemotherapeutic agents in CRC patients.

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