Capecitabine treatment of HCT-15 colon cancer cells induces apoptosis via mitochondrial pathway

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Abstract

**Purpose**: To investigate the effect of capecitabine on apoptosis induction in HCT-15 colon carcinoma cells and investigate the underlying mechanism.

**Methods**: Phase-contrast microscopy was used for the examination of morphological changes while flow cytometry was employed for the analysis of cell cycle distribution, induction of apoptosis, reactive oxygen species (ROS) production and expression of caspases. Western blot assay was used for the analysis of expression level of apoptosis-related and cell cycle regulatory proteins.

**Results**: Capecitabine treatment caused changes in the morphological appearance of HCT-15 cells after 48 h. The viability of HCT-15 cells was reduced to 23% on treatment with capecitabine (5 μM) compared to 98% in the control cultures. Incubation with capecitabine increased the population of HCT-15 cells in G0/G1 phase to 56.43% compared to 41.67% in the control. Capecitabine treatment of HCT-15 cells caused condensation of DNA and induced apoptosis in a concentration-dependent manner. At 5 μM concentration of capecitabine, apoptosis was induced in 45.74% of the cells. Incubation of HCT-15 cells with capecitabine for 48 h led to a significant increase in the production of ROS. Translocation of Endo G and AIF from mitochondria to the nuclei increased significantly (p < 0.005) on treatment with 5 μM capecitabine. Capecitabine treatment also reduced the expression of cyclin E and Cdc25c and promoted the level of caspases, Bax, AIF, Endo G, p21, PARP and p-p53. The expression level of Bcl-2 decreased in HCT-15 cells on incubation with 5 μM concentration of capecitabine.

**Conclusion**: Capecitabine treatment causes inhibition of colon cancer growth via the mitochondrial pathway of apoptosis. Thus, capecitabine may have therapeutic application in colon carcinoma treatment.

**Keywords**: Capecitabine, 5-Fluorouracil, Translocation, Colon cancer, Colitis, Apoptosis

INTRODUCTION

Colorectal cancer is globally considered as the most common cancer found in people of all age groups and alone constitutes 10 % of cancer-related deaths [1]. It is a malignant neoplasm originating from the lining of the colon. Colorectal cancer metastasizes to various distant organs and its most common target is the liver. One type of colon cancer is colitis-associated cancer which is characterized by the onset of inflammatory bowel disease of ulcerative colitis [2]. It has been reported that 15 % of patients with colorectal cancer show liver metastases [3]. Among various types of solid tumors only in patients with colorectal cancer can the survival term be increased by surgical extraction of distant metastases from various organs such as liver...
and lungs [4]. The survival of the unresectable metastases of colorectal cancer patients can be increased by treatment with systemic chemotherapy [5].

Cell apoptosis is a programmed mechanism for death of unwanted cells which can take place via intrinsic or extrinsic pathways [6,7]. Various ligands interact with surface receptors (tumor necrosis factor) and death receptors leading to cell apoptosis through extrinsic pathway [7,8]. However, cellular processes like oxidative stress, DNA damage and cytotoxic agents lead to apoptosis through the intrinsic pathway [7].

In caspase dependent method of apoptosis various proteins such as components of Bcl-2 class are involved in the maintenance of activation of caspases [9,10]. Treatment of cancer by induction of cell apoptosis using chemotherapeutic agents is one of the best strategies. Therefore, screening of new molecules for the development of treatment strategy can improve the clinical outcome for patients with colorectal cancer.

Capecitabine is a modified derivative of fluoropyrimidine aimed to selectively transfer 5-fluorouracil into tumor cell. The method of administration of capecitabine is very simple and comfortable. In gastric cancer treatment capecitabine is used either singly or in combination with other chemotherapeutic agents [11]. Administration of higher doses of capecitabine to patients of gastric cancer cause side effects such as diarrhea, lymphopenia, nausea etc. However, lower doses have been found to be effective.

In the present study effect of capecitabine on viability and apoptosis induction in human colon cancer cells was investigated. The study demonstrated that capecitabine treatment at lower doses causes inhibition of colon cancer growth via mitochondrial pathway of apoptosis. Thus capecitabine can be a very good candidate for the treatment of colon cancer.

**EXPERIMENTAL**

**Chemicals and reagents**

Capecitabine was purchased from Roche Pharmaceutical Company (Nutley, NJ, USA) and its solution was freshly prepared at the time of use in dimethylsulfoxide (DMSO). Propidium iodide, Triton X-100, Tris-HCl and trypan blue were supplied by Sigma Chemical Co (St Louis, MO, USA).

**Cell culture and cell viability assay**

HCT-15 human colon cancer cell line purchased from Food Industry Research and Development Institute (Hsinchu, Taiwan). The cell cultures were grown in RPMI-1640 (Gibco BRL) containing 10 % fetal bovine serum (FBS, HyClone). The medium was supplemented with 2 mM glutamine (Sigma-Aldrich) and antibiotics such as penicillin (100 U/mL) and streptomycin (100 μg/mL). The cells were cultured in an incubator at 37 °C temperature under 5 % CO₂ atmosphere.

**Determination of cell morphology and viability**

HCT-15 cells at 4 x 10^4 cells per well density were distributed in 12-well plates and incubated for 24 h. The cells were then subjected to 48 h incubation with 0, 1, 2, 3, 4 and 5 μM doses of capecitabine under an atmosphere of 5 % CO₂ at 37 °C. After incubation phase contrast microscope was used to examine the cells for changes in morphology. For analysis of viability the cells were collected after incubation with capecitabine, centrifuged and then subjected to PI (5 μg/mL) staining before analysis using flow cytometry (Becton-Dickinson, San Jose, CA, USA).

**Analysis of apoptosis**

HCT-15 cells after incubation with or without capecitabine for 48 h were collected and trypsinized. The cells were then washed twice with phosphate-buffered saline and subsequently resuspended at a density of 2 x 10^6 cells per ml in binding buffer.

The cells were then treated with 5 μL each of annexin-V-fluorescein isothiocyanate and propidium iodide (PI) and subjected to incubation for 15 min at room temperature. FACScalibur Flow Cytometer (BD Biosciences, San Jose, CA, USA) was used for the analysis of cell apoptosis.

**Analysis of cell cycle**

HCT-15 cells after incubation with or without capecitabine for 48 h were collected and trypsinized. The cells were collected and then fixed overnight at 4 °C using 70 % ethyl alcohol. The cells were re-suspended in the dark at 37 °C for half an hour in PBS supplemented with PI (40 μg/mL) and RNase (0.1 mg/mL) Triton X-100 (0.1 %). Flow cytometer connected with an argon
ion laser was used for analysis of the cells at a wavelength of 490 nm.

**Determination of reactive oxygen species (ROS) production**

HCT-15 cells at density of 2 x 10^6 cells per mL were incubated with indicated doses of capecitabine for 48 h. The cells were collected and treated with 10 µM solution of 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA) for half an hour at 37 °C. After incubation the cells were analyzed using flow cytometry for production of reactive oxygen species.

**Western blot analysis**

HCT-15 cells after incubation with capecitabine for 48 h under the appropriate conditions were collected and washed two times with ice-cold PBS. The cells were then treated with lysis buffer [40 mM Tris (pH 8.0), sodium chloride (120 mM), NP-40 (0.5 %), sodium or thovanadate (0.1 mM), aprotinin (2 µg/mL), leupeptin (2 µg/mL) and phenymethylsulfonyl fluoride (100 µg/mL)] to get the lysate. From the lysate supernatant was collected to determine the concentration of proteins (Pierce, Rockford, IL).

Denaturation of extracted proteins was performed with sample buffer [Tris-HCl (0.5 M), pH 6.8, 4 % SDS, glycerol (20 %), bromophenol blue (0.1 %), β-mercaptoethanol (10 %)] for 10 min at 100 °C. Isolation of the proteins was achieved on 6-15 % SDS-PAGE and the proteins were subsequently transferred onto PVDF membranes.

The non-specific sites in the membranes were blocked for 1 h with 5 % non-fat dry milk in Tris-buffered saline with Tween-20 buffer at room temperature. Membrane incubation with primary antibodies was performed overnight. Following incubation 4 X TBS-T buffer was used for washing of the membrane for 15 min followed by 1 h incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

Thereafter, 1 X TBS-T buffer was used for washing the membrane for 15 min. The complex format on interaction of antigen with antibody was analyzed using enhanced chemiluminescence (ECL) detection system (GE Healthcare Biosciences, Pittsburgh, PA). The primary antibodies against caspase-9, -3, -8, Bax, Bal-2, Endo G, AIF and PARP were obtained from R&D Systems (Minneapolis, USA) whereas those against Cdc25c, cyclin E, p-

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**Confocal laser scanning microscopy for protein translocation**

HCT-15 cells were distributed at a density of 2 x 10^6 cells per well onto 4-well chamber slides and cultured. The cells were incubated without or with the indicated concentration of capecitabine for 48 h. Cells on the slides were fixed for over 20 min using 4 % formaldehyde in PBS. The cells were permeabilized by treatment with Triton-X 100 (0.3 %) in PBS for 1 h. The non-specific sites were blocked with 2 % BSA. Staining of the cells was performed overnight by incubation with primary antibodies against AIF and Endo G (1 : 100 dilution). The stained cells were incubated with secondary antibody (FITC conjugated goat anti-mouse IgG at 1 : 100 dilution) and subsequently subjected to DNA staining using mitotracker. Leica TCS SP2 confocal spectral microscope was used for obtaining the photomicrographs.

**DAPI staining**

HCT-15 cells at a density of 2 x 10^6 cells per mL were incubated with or without capecitabine for 48 h. After incubation the cells were subjected to DAPI (4, 6-diamidino-2-phenylindole dihydrochloride) staining. Then fluorescence microscope was used for examination of the stained cells.

**Statistical analysis**

The data are expressed as mean ± standard deviation (SD). Student’s t-test was used for the analysis of the differences among the groups; p < 0.05 was considered statistically significant. Microsoft Excel 2007 was used for the analysis

**RESULTS**

**Capecitabine alters the morphology and reduces viability of HCT-15 cells**

Phase-contrast microscopy showed changes in the morphological appearance of HCT-15 cells on incubation with capecitabine. Changes in the cell morphology were dependent on the concentration of capecitabine. Flow cytometry revealed that capecitabine treatment significantly (p < 0.005) reduced the viability of HCT-15 cells. At 5 µM concentration of capecitabine the viability of HCT-15 cells was reduced to 23 % compared to 98 % in the control cultures (Figure 1B).
Figure 1: Capecitabine alters the morphology and reduces the viability of HCT-15 cells. Incubation of the cells with 1, 2, 3, 4 and 5 μM concentrations of capecitabine was carried out for 48 h. (A) Changes in cell morphology was examined using phase-contrast microscopy after 48 h of treatment. (B) The cell viability was analyzed using flow cytometry; *p < 0.05 compared to the control cells.

Capecitabine increases the population of HCT-15 cells in G0/G1 phase

Incubation of HCT-15 cells with capecitabine for 48 h increased the population of cells in G0/G1 phase in a concentration dependent manner with subsequent reduction in S-phase (Figure 2). Capecitabine treatment at 5 μM concentration increased the population of HCT-15 cells in G0/G1 phase to 56.43 % compared to 41.67 % in the control. The population of cells in S-phase was decreased to 19.89 % in capecitabine treated cultures compared to 33.73 % in the control cultures.

Figure 2: Capecitabine arrests cell cycle of HCT-15 cells. Cell incubation for 48 h with 1, 2, 3, 4 and 5 μM of capecitabine was followed by analysis using flow cytometry; *p < 0.05 in comparison with control.
Capecitabine treatment of HCT-15 cells causes induction of apoptosis

The results from DAPI and Annexin V staining revealed that capecitabine treatment of HCT-15 cells caused condensation of DNA and induced apoptosis in concentration dependent manner (Figure 3). At 5 μM concentration of capecitabine apoptosis was induced in 45.74 % of HCT-15 cells after 48 h compared to 3.39 % in the control.

Capecitabine increases reactive oxygen species production (ROS) in HCT-15 cells

Incubation of HCT-15 cells with various concentrations of capecitabine for 48 h led to increase in the production of ROS in concentration dependent manner. The production of ROS was significantly higher at 5 μM concentration of capecitabine after 48 h (Figure 4).

Capecitabine increases translocation of Endo G and AIF from mitochondria in colon carcinoma cells

Incubation of HCT-15 cells for 48 h with capecitabine increased the translocation of Endo G and AIF from mitochondria to the nuclei. The expression level of Endo G and AIF proteins was significantly (p < 0.005) higher in HCT-15 cells at 5 μM capecitabine (Figure 5).

Figure 3: Capecitabine induces apoptosis in HCT-15 cells. The cells after incubation for 1, 2, 3, 4 and 5 μM concentration of capecitabine were collected and stained with DAPI and Annexin V for analysis of DNA damage and induction of apoptosis; *p < 0.05 compared to control.
Figure 4: Capecitabine increases reactive oxygen species production (ROS) in HCT-15 cells. The cells were incubated with 1, 2, 3, 4 and 5 μM concentration of capecitabine for 48 h and then the production of ROS was analysed. DCFH-DA stain was used for flow cytometric examination; \( p < 0.05 \) compared to control.

**Capecitabine alters expression of cell cycle and apoptosis-related proteins in HCT-15 cells**

Western blot analysis showed that incubation of HCT-15 cells with various concentrations of capecitabine influenced the expression of p21, p-p53, cyclin E, Cdc25c, Bax and cleavage-caspases after 48 h. HCT-15 cells on incubation with 5 μM doses of capecitabine reduced the expression of cyclin E and Cdc25c and promoted the level of caspases-8, -3, Bax, AIF, Endo G, PARP, p21 and p-p53. The expression level of Bcl-2 was decreased in HCT-15 cells on incubation with 5 μM capecitabine for 48 h (Figure 6).

**DISCUSSION**

The current study was aimed to investigate the effect of various concentrations of capecitabine on HCT-15 human colon cancer cells. The results showed a decrease in viability and increase in apoptosis of HCT-15 cells via mitochondrial pathway on exposure to capecitabine.
Incubation of HCT-15 cells with capecitabine led to reduction in viability to a marked level based on the concentration used. Progression of cell cycle through various phases is regulated by different kinases which play important role in the action of cdc/cdk kinases [12]. Cyclins D/cdk on activation regulate progression of G1, E/cdk2 activation G1/S, A/cdk2 activation S and cyclin A and cyclin B/cdk1 regulate progression of G2/M phases [13, 14]. Investigation of the underlying mechanism of capecitabine induced reduction in HCT-15 cell viability revealed arrest of cell cycle in G0/G1 phase. TheG0/G1 phase arrest was further confirmed by increase in p53 and p21 expression and decrease in Cdc25c and cyclin E level in capecitabine treated cells. Up-regulation of Bax expression causes increase in the mitochondrial membrane permeability which results in activation of caspases and subsequently cell apoptosis [15-17]. In HCT-15 cells incubation with capecitabine induced apoptosis significantly and increased the activation of caspases. The mitochondrial translocation of proteins AIF and Endo G was increased significantly on incubation of HCT-15 cells with capecitabine. The level of pro-apoptotic protein (Bax) expression was promoted in the cells incubated with capecitabine. On the other hand, the anti-apoptotic protein Bcl-2 expression was decreased. It is reported that increase in the generation of cellular reactive oxygen species induces cell cycle arrest and apoptosis [18, 19]. Results from the current study showed that incubation of HCT-15 cells with capecitabine led to increase in the production of reactive oxygen species. There are reports that increase in the expression level of p21 in the cells is related with the JNK pathway activation [20]. The results from the present study showed that capecitabine treatment of HCT-15 cells significantly increased the expression of p21.

CONCLUSION

Capecitabine treatment causes inhibition of colon cancer growth via the mitochondrial pathway of apoptosis. Thus, capecitabine may be useful for the treatment of colon cancer.

DECLARATIONS

Acknowledgement

None declared

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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