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Research Article

Targeting ceramidase and sphingosine kinase 1 of ceramide metabolic pathway induces apoptosis in human colon cancer cells

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Abstract

The present study investigates the apoptotic effect of ceramidase inhibitor, Ceranib 2 and Sphingosine kinase 1 inhibitor, Dimethyl sphingosine in human colon cancer cell line SW480. Cells were treated with different doses of Ceranib 2 and Dimethyl sphingosine for 24, 48 and 72 hours to determine the cell viability using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT assay). Morphological changes were examined in phase contrast microscope, nuclear changes were observed using DAPI staining. DNA gel electrophoresis was performed to visualize DNA changes, Acridine orange/Ethidium bromide staining was done to discriminate live, early apoptotic, late apoptotic, necrotic cells. Mitochondrial membrane potential was determined by JC-1 staining. MTT assay revealed the cytotoxic effect of Ceranib 2 and Dimethyl sphingosine in SW480 cell line in dose and time dependant manner. Morphological changes and chromatin condensation observed in DAPI staining are morphological hallmarks of apoptosis. DNA ladder pattern observed in drug treated groups with respect to control group is biochemical hallmark of apoptosis. Further, presence of early and late apoptotic cells in Acridine orange/Ethidium bromide staining, loss of mitochondrial membrane potential in drug treated groups confirmed the apoptotic effect of Ceranib 2 and Dimethyl sphingosine in SW480 cells.

Keywords: *Colon cancer, Apoptosis, Ceranib 2, Dimethyl sphingosine, Ceramidase, Sphingosine kinase 1*

1. Introduction

Colon cancer or colorectal cancer is characterized by uncontrolled proliferation of cells in large intestine of which 90% are adenocarcinomas originates from epithelial cells of colorectal mucousa (Hamilton, *et al*, 2010). As per 2017 estimates colon cancer is expected to be 9% of all the cancers in men and 8% of all the cancers in women worldwide. Further among men the expected mortality rate ranks next to lung cancer (Seigel, *et al*, 2017). Though numerous chemotherapeutics are available for the treatment of colon cancer the major problem encountered is the development of resistance after prolonged treatment and their ability to metastasize to liver (70%), thorax (32%) and peritoneum (21%) (Riihimaki, *et al,* 2016).

Recent research on colon cancer explored the essential role of ceramides and sphingosine-1-phosphate in the regulation of cell death and resistance (Ponnusamy, *et al*, 2010). Ceramide and Sphingosine-1-phosphate are the key metabolites of ceramide metabolic pathway. Ceramide, the pro-apoptotic molecule is metabolized to sphingosine by ceramidases which is further phosphorylated to sphingosine-1-phosphate by sphingosine kinase 1. Sphingosine-1-phosphate signals for cell proliferation, cell survival and angiogenesis (Brizuela, *et al*, 2014), (Harita, *et al*, 2014). Further, induction of de novo ceramide synthesis in colon cancer cells was effective against drug resistant colon cancer cells (Panarian, *et al*, 2008), (Schiffmann, *et al*, 2009). Interestingly, ceramide levels in primary and metastatic color cancer patient specimens were lower than their normal mucosa (Selzner, *et al,* 2001).Moreover, 89% of colon cancers showed higher expression of sphingosine kinase 1 than normal colon mucosa (Kawamori, *et al*, 2009). Therefore we aimed at targeting ceramidase and sphingosine kinase 1 to induce apoptosis of colon cancer cells. Inhibition of ceramidase with an inhibitor prevents the catabolism of ceramide whereas, inhibition of sphingosine kinase 1 abrogates the synthesis of sphingosine-1-phosphate thereby promotes cell death.

Ceranib 2 was used as Ceramidase inhibitor and Ceranib 2 was reported to induce apoptosis in human ovarian carcinoma cells (Draper, *et al*, 2011), human breast cancer cells (Vethakanraj, *et al,* 2015) and rat fibroblasts (Vejselova, *et al*, 2014). Dimethyl sphingosine was used as sphingosine kinase 1 inhibitor and Dimethyl sphingosine significantly induced

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apoptosis of human lung cancer cells (Chen, et al, 2014) and prostate cancer cells (Nava, et al, 2000). But, the effect of Ceranib 2 and Dimethyl sphingosine in colon cancer cells is elusive. In the present study, we evaluated the apoptotic effect of Ceranib 2 and Dimethyl sphingosine in colon cancer cells.

2. Materials and Methods

2.1 Cell culture

Human colon cancer cell line SW480 was procured from National Centre for Cell Sciences, Pune, India and the cells were grown in Dulbecco's Minimum essential medium (Sigma Aldrich, USA) supplemented with 10% fetal bovine serum (Gibco, USA), Penicillin (100 U/ml) and Streptomycin (100 µg/ml). The cells were maintained at 37° C in an incubator with 5% CO_2. The cells were passaged with trypsin-EDTA after attaining 80% confluency.

2.2 Cell viability assay

To determine the cytotoxic effect of Ceranib 2 and Dimethyl sphingosine cell viability assay was performed using MTT (Sigma Aldrich, USA). 1 x 10^4 cells of SW480 were seeded in 96 well flat bottomed tissue culture plates with complete culture medium. The cells were incubated in CO_2 incubator overnight and after attachment they were treated with different doses of Ceranib 2 and Dimethyl sphingosine in the medium containing 5% fetal bovine serum for 24, 48 and 72 hours. After treatment MTT was added to each well and incubated at 37°C for 3 hours and the formazan crystals were dissolved in Dimethyl sulphoxide. The absorbance was measured at 570 nm in ELIZA reader. The percentage viability was calculated using the formula (A_{570} test/ A_{570} control) X 100%.

2.3 Morphological Examination

To study the morphological changes associated with drug treatments the cells were seeded in 24 well plate and treated with IC_{50} doses of Ceranib 2 and Dimethyl sphingosine for 24 hours. The changes were observed under phase contrast microscope at 200X magnification and photographed.

2.4 DAPI staining

DAPI staining was performed to determine the nuclear changes in drug treated groups. SW480 cells were seeded in 24 well plate and after attachment cells were treated with IC_{50} doses of Ceranib 2 and Dimethyl sphingosine for 24 hours. After treatment the medium was removed completely, the cells were fixed with 100% methanol at -20° C for 10 minutes and incubated with DAPI at room temperature for 10 min. The cells were washed with PBS and visualized under fluorescent microscope at an excitation of 359 nm and emission of 451 nm.

2.5 DNA gel electrophoresis

DNA gel electrophoresis was performed to visualize the DNA changes observed in apoptotic cells. SW480 cells were treated with IC_{50} doses of Ceranib 2 and Dimethyl sphingosine for 24 hours. The cells were pelleted and DNA samples were isolated from control and treated groups using Qiagen mammalian genomic DNA isolation kit. The DNA samples were separated in 1.2% agarose gel electrophoresis and visualized under UV trans illuminator and photographed.

2.6 Acridine Orange/ Ethidium bromide staining

Acridine Orange/ Ethidium bromide staining was done to discriminate live, early apoptotic, late apoptotic and necrotic cells in drug treated groups. The cells were treated with IC_{50} doses of Ceranib 2 and Dimethyl sphingosine for 24 hours. The cells were stained with Acridine orange (100 µg/ml) and Ethidium bromide (100 µg/ml), visualized under fluorescent microscope at 200X magnification.

2.7 JC-1 staining

JC-1 staining was performed to assess the mitochondrial membrane potential of control and drug treated groups. SW480 cells were seeded in 24 well plate and after attachment they were treated with IC_{50} doses of Ceranib 2 and Dimethyl sphingosine for 24 hours. JC-1 dye was added to the wells and incubated in CO_2 incubator for 15 min. Red and green signals were visualized under fluorescent microscope and photographed after merging the images. JC-1 aggregates (red signals) were detected at excitation/ emission = 540/570 nm, JC-1 monomers (green signals) were detected at excitation/emission = 485/535 nm.

Statistical Analysis

The data were expressed in mean ± SD and statistical analyses were performed using two-way ANOVA for comparison between control and treatment groups using GraphPad Prism 5 software. P value < 0.05 was considered to be significant.

3. Results

3.1 Effect of Ceranib 2 and Dimethyl sphingosine in the viability of SW480 cells

Ceranib 2 and Dimethyl sphingosine induced cell death of SW480 cells in dose and time dependant manner. IC_{50} attained at 25 µM, 15 µM, 7.5 µM after 24, 48 and 72 hours of treatment with Ceranib 2 (Fig. 1) whereas, IC_{50} attained at 5 µM, 2.5 µM, 1 µM after 24, 48 and 72 hours of treatment with Dimethyl sphingosine (Fig. 2). Thus Dimethyl sphingosine was found to be more

potent in inducing cell death of SW480 cells than Ceranib 2. IC_{50} doses of 72 hours were standardized for further assays.

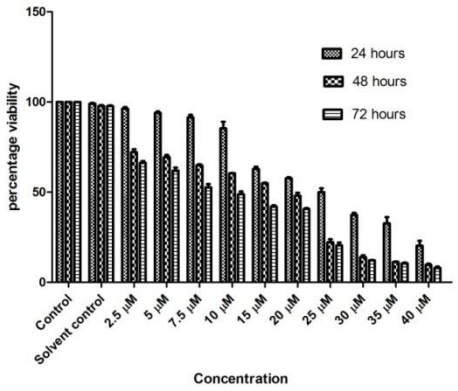

Fig.1 Effect of Ceranib 2 on SW480

3.2 Effect of Ceranib 2 and Dimethyl sphingosine in the morphology of SW480 cells

Morphological changes observed under phase contrast microscope at 400X (top panel) and 200X (bottom panel) magnifications are shown in Figure 3. In untreated SW480, no morphological change was observed whereas in the cells treated with Ceranib 2 and Dimethyl sphingosine the cells became rounded and detached from the surface of the plate.

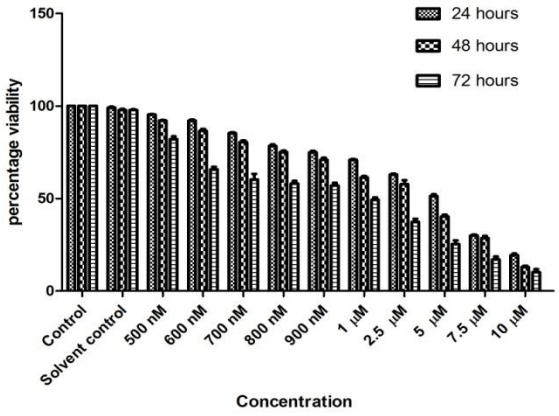

Fig.2 Effect of Dimethyl sphingosine on SW480

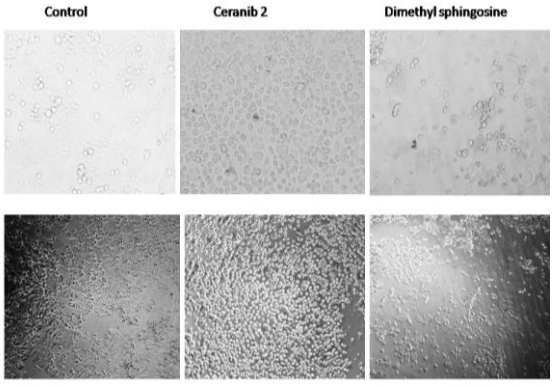

Fig.3 Morphological changes after drug treatment

3.3 DAPI staining to detect nuclear changes

In control cells, the staining was uniform but in drug treated groups bright stained nuclei indicating chromatin condensation was clearly visible. Chromatin condensation is a morphological hallmark of apoptosis and more number of condensed nuclei were observed in Dimethyl sphingosine treated group than Ceranib 2 treated group (Fig. 4)

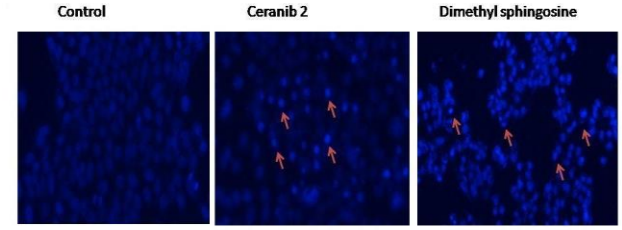

Fig.4 Nuclear changes after drug treatment

3.4 DNA gel electrophoresis

DNA fragmentation is a biochemical hallmark of apoptosis and is characterized by ladder pattern of DNA in apoptotic cells. Treatment with Ceranib 2 (lane 3) and Dimethyl sphingosine (lane 6) significantly induced DNA fragmentation in SW480 but in control group (lane 2 and 5) the DNA remained intact (Fig. 5)

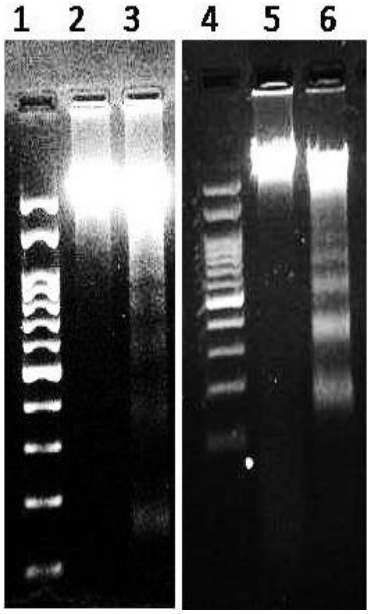

Fig.5 DNA gel electrophoresis

3.5 Acridine orange/Ethidium Bromide to discriminate live, early apoptotic, late apoptotic, necrotic cells

Both live and dead cells take up Acridine orange stain and fluoresce green but dead cells take up ethidium bromide stain and fluoresce red. Live cells appear green, early apoptotic cells also appear green but possess condensed and fragmented chromatin (red arrows). Late apoptotic cells and necrotic cells appear

orange (blue arrows). The apoptotic changes observed under fluorescent microscope at 400X and 200X are shown in Figure 6. Cell shrinkage, chromatin condensation, fragmentation and late apoptotic bodies observed in drug treated groups indicate the apoptosis inducing ability of Ceranib 2 and Dimethyl sphingosine in SW480 cells.

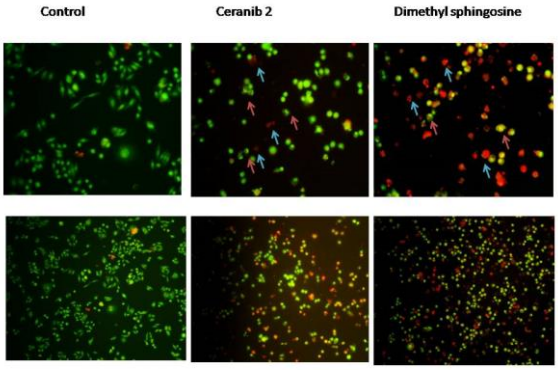

Fig.6 Acridine orange/ Ethidium bromide staining

3.6 JC 1 staining to assess mitochondrial membrane potential

JC 1 is a cationic dye and it accumulates as JC 1 monomers in the areas of normal polarized mitochondrial membrane and fluoresce red but it accumulates as JC 1 aggregates in the areas of depolarized mitochondrial membrane and fluoresce green. The decreased red fluorescence in Ceranib 2 and Dimethyl sphingosine treated groups with respect to control group indicate the loss of mitochondrial membrane potential in drug treated groups (Fig. 7).

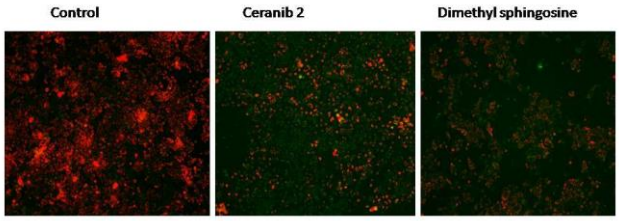

Fig.7 JC 1 staining

Discussion

Ceramidase and sphingosine kinase 1 of ceramide metabolic pathway play a key in ceramide/sphingosine-1-phosphate rheostat (Baran, *et al,* 2007). In colon cancer cells, induction of denovo ceramide synthesis selectively increased C_{16}, C_{24}, $C_{24::1}$ ceramide concentrations and promoted cell death (Schiffmann, *et al*, 2009). Therefore, targeting ceramidase enzyme to prevent ceramide degradation is one of the strategies of colon cancer treatment. In addition, sphingosine kinase 1 being over expressed in colon cancer plays an inevitable role in colon cancer metastases (Kawamori, *et al*, 2009). Targeting sphingosine kinase 1 to abrogate sphingosine-1-phosphate generation is another potential strategy in treating colon cancer. In this study, we have used Ceranib 2 as ceramidase inhibitor and Dimethyl sphingosine as sphingosine kinase 1 inhibitor to induce apoptosis of colon cancer cell line SW480.

Cell viability assay revealed the cytotoxic effect of Ceranib 2 and Dimethyl sphingosine in SW480 cells. Though both the drugs were effective in inducing cell death Dimethyl sphingosine with an IC_{50} of 1 µM was found to be more potent in inhibiting the growth of SW480 cells. Cell shrinkage and detachment of cells observed under phase contrast microscope, chromatin condensation and fragmentation observed under DAPI, AO/EtBr staining are morphological hallmarks of apoptosis (Kerr, *et al,* 1994), (Kerr, *et al,* 1972). These morphological events are due to cleavage of nuclear and cytoskeletal proteins such as PARP, fodrins, Numa by activated proteases (Enari, *et al,* 1998).

DNA fragmentation observed under gel electrophoresis after Ceranib 2 and Tamoxifen treatment is biochemical hallmark of apoptosis (Wyllie, 1980). This event is characterized by endogenous DNAases which cut internucleosomal regions into double stranded DNA of 180-200 bps (Saraste and Pulkki, 2002) DNA fragmentation factor (DFF40) (Liu, *et al,* 199) and caspase activated DNAases (CAD) ((Enari, *et al,* 1998), (Sakahira, *et al,* 1998) are responsible for the fragmentation during apoptosis. In normal cells, DFF40 and CAD are associated with inhibitor proteins DFF45 (Liu, *et al,* 1997) and ICAD (inhibitor CAD) (Sakahira, *et al,* 1998). In apoptotic cells, these enzymes are activated by active caspases resulting in the formation of double stranded DNA breaks (Liu, *et al*, 1997), Sakahira, *et al,* 1998), (Tang and Kidd, 1988)

Loss of mitochondrial membrane potential in drug treated groups with respect to control group is another critical event in apoptosis (Gottlieb, *et al*, 2003). Loss in mitochondrial membrane potential occurs due to formation of mitochondrial membrane transition pore and as result releases cytochrome C from the inter membrane space of mitochondria into cytoplasm (Liu, *et al.* 1996). Cytochrome C triggers the activation of caspases and mediates apoptosis (Achen, *et al*, 2002).

To conclude, targeting ceramidase and sphingosine kinase 1 enzymes of ceramide metabolic pathway significantly induces apoptosis of colon cancer cell lines. Further, the apoptotic effect was more pronounced after sphingosine kinase 1 inhibition than ceramidase inhibition. As inhibition of ceramidase and sphingosine kinase 1 induced apoptosis, we suggest these enzymes as potential targets for colon cancer.

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