Effect of 5-Flourouracil On HCT-116 P53+/- Human Colon Cancer Cells.

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ABSTRACT

The purpose of this study was to observe the effects of 5-flourouracil on HCT-116 p53 +/- colon cancer cells. In order to investigate the effects of the drug, concentration-dependent assays and time-dependent assay procedures were used. To evaluate the cell viability after being treated with the drug, XTT technique was used. After performing concentration-dependent assays a consequent decrease in cell viability was observed with increase in concentration of the drug. The time-dependent assay showed a gradual decrease in cell viability with increase in time after exposure to the drug. Western blotting technique was performed on the proteins extracted from the samples. Expression of Bak was examined after the cells were exposed to the drug. Results from western blotting technique revealed that Bak protein was initially over-expressed after the cells were treated with 5-Flourouracil for 48 hours and this over-expression of the Bak resulted in apoptosis of cancer cells. Thus, 5-Flourouracil is considered to be an effective chemotherapeutic drug for colon cancer cells partially though the over-expression of Bak protein.

Key Words: 5-Flourouracil, HCT-116p53+/-, cell viability, Bak protein.
# Table of Contents

1. Introduction ........................................................................................................... 1  
   1.1 Colorectal cancer ............................................................................................ 1  
   1.2 Chemotherapy ............................................................................................... 3  
   1.3 5-Flourouracil ............................................................................................... 4  
   1.4 Antibody ....................................................................................................... 5  
   1.5 HCT116 p53 cell line ..................................................................................... 6  
2. Aim .................................................................................................................... 6  
3. Materials and methods ....................................................................................... 6  
   3.1 Chemicals and Cell Culture .......................................................................... 6  
   3.2 Experimental design ...................................................................................... 7  
      3.2.1 Concentration – Dependent Assay ........................................................ 7  
      3.2.2 Time Dependent Assay ......................................................................... 7  
      3.2.3 XTT Assay .............................................................................................. 8  
   3.3 Western blotting ............................................................................................ 8  
      3.3.1 Protein Extraction Reagent ................................................................... 8  
      3.3.2 Protein Extraction .................................................................................. 9  
      3.3.4 Antibodies ............................................................................................ 9  
4. Results .............................................................................................................. 11  
   4.1 Concentration dependent assay ...................................................................... 11  
   4.2 XTT concentration dependent curve ............................................................... 13  
   4.3 XTT time dependent curve ............................................................................ 14  
   4.4 Western blotting ........................................................................................... 15  
5. Discussion ......................................................................................................... 17  
6. Conclusion ......................................................................................................... 17  
7. References ......................................................................................................... 19  
Appendix ................................................................................................................ 23
List of Figures

Figure 1: Shows anatomical position of colon and rectum ................................................. 2
Figure 2: Shows examination of colon and sigmoid by mean of colonoscopy and sigmoidoscopy .......................................................................................................................... 4
Figure 3: Shows chemical structure of 5-Fluorouracil ......................................................... 4
Figure 4: shows the cells in the control wells in A at 24 hours of incubation and B at 48 hours of incubation thus showing that the number of cells increase with passage of time after incubation in the control well. ......................................................................................... 11
Figure 5: shows the microscopic observation of cells treated with different concentration of drug that is 25 µM, 50 µM, 75 µM and 100 µM in figure A, B, C and D respectively. The cell number is more when treated with lowest concentration of drug 25 µM (figure 5A), cell concentration is less when treated with maximum concentration 100 µM (figure 5D). .................................................................................................................................. 12
Figure 6: Represents XTT Concentration dependent Graph ................................................. 13
Figure 7: XTT Time dependent Graph .................................................................................. 14
Figure 8: Shows bands for β-Actin and Bak ........................................................................ 15
Figure 9: Diagrammatic comparison of expression of Bak and β-Actin antibodies.............. 16
Figure 10: Shows standard curve for protein extraction ..................................................... 26

List of Tables

Table 1: Following antibodies were used in western blotting............................................. 9
Table 2: The following table represents the concentration of protein extracted from human colon cells both control and treated for 24hour, 48hours and 72hours......... 14
Table 3: Shows the percentages of gels and their composition ......................................... 25
1. Introduction

Cancer occurs due to abnormal cellular proliferation resulting in tumor formation. The rate of cell proliferation overcomes the rate of apoptosis, thus resulting in abnormal growth. Three types of genes are involved in tumor formation,

- Oncogenes
- Tumor suppressor gene
- DNA repair genes

Cellular proliferation is regulated by p53 pathway and in-order to maintain genomic integrity it also responds to DNA damage (Obata, Kominami and Mishima, 2012). Increase in DNA damage and reduced activity of DNA repair genes are thought to be markers in carcinogenesis (Slyskova et al., 2012). Polymorphism or alteration in genes that are involved in DNA repair systems are correlated with the occurrence of colorectal cancer (Obata, Kominami and Mishima, 2012). Genes that are involved in DNA repair systems are targets for chemotherapeutic drugs (Michailidi, Papavassiliou and Troungos, 2012).

1.1 Colorectal cancer

Gastrointestinal tumors are second worldwide leading cause of death (MacKenzie, Spithoff and Jonker, 2011).
Figure 1: Shows anatomical position of colon and rectum (Colon Cancer (Colorectal Cancer) Information, Causes, Symptoms, Prevention and Treatment on MedicineNet.com, n.d.)

Figure 1 shows different parts of colon. The colon is divided into following parts:

- The ascending colon travel up the right side of abdomen.
- The transverse colon runs across the abdomen.
- The descending colon travels down the left abdomen.
- The sigmoid colon is a short curving of the colon, just before the rectum.
- The last part of colon is termed as rectum.

Cancer involving colon and rectum are termed as colorectal cancer. Colorectal cancer is the most prevailing tumors worldwide (Oliveira, Machado, Sabbaga and Hoff, 2010). It is third most common cause of death due to cancer (Michailidi, Papavassiliou and Troungos, 2012). Tumors of gastrointestinal tract are aggressive usually with poor outcomes of survival (Sohal and Sun, 2011). Colorectal carcinoma might be familial (Persson et al., 2012). The common hereditary form of colorectal cancer is Lynch syndrome occurring due to mutation in mismatch repair genes (Schneider et al., 2012). MYH gene mutation leads to an inherited form of colorectal cancer (Laarabi et al., 2012). Polyunsaturated fatty acids also increase risk of developing colorectal cancer (Cai, Dupertuis and Pichard, 2012). Mutations in germline lead to autosomal recessive form of colorectal cancer (Laarabi et al., 2012). Sometimes toxic megacolon which is an Inflammatory Bowel Disease also leads to obstructive form of colorectal cancer (Autenrieth and
Baumgart, 2012). Researchers are being done to evaluate the risk of developing colorectal cancer among people living in places near to metal industries (Lu, Zhu, Lu and Chi, 2007).

1.2 Chemotherapy

A lot of development has been observed in the treatment of colorectal cancer since 1990. Combination therapies have been used vastly in treating colorectal carcinomas (Takiuchi, 2010). People taking chemotherapy suffer from reduced appetite and distressing altered food sensations (Boltong and Keast, 2012). Usually colorectal carcinoma ends up with poor prognosis and patients do end up with resections and palliative measures (Glimelius and Cavalli-Björkman, 2012). The use of chemotherapy results in the relief of clinical symptoms of patients suffering from colorectal carcinoma. Patients suffering from advanced stages of colorectal carcinoma are found in good state maybe due to the use of adjuvant therapy (Popov, Jelić, Radosavljević and Nikolić-Tomasević, 1998). Before starting treatment it is essential to differentiate between benign and malignant neoplasm (Blend and Bhadkamkar, 1998). Colorectal carcinoma metastasizes to liver, however chemotherapies and surgical options have been advented to overcome the hepatic metastasis being caused by colorectal carcinoma (Yoo, Lopez-Soler, Longo and Cha, 2006). Colonoscopy is considered to be a diagnostic tool for identifying colonic diseases and on the other hand endoscopic treatment is the easiest method to treat simple colorectal neoplasm (Su et al., 2005).
Colonic diseases have been revolutionized with the advent of flexible sigmoidoscopy and colonoscopy (figure 2). Sigmoidoscopy helps to view lower part of colon while colonoscopy helps to view both upper and lower sections (figure 2). Colonoscopy is becoming more important because of its great utilization in screening of colon cancer (Cappell and Friedel, 2002).

1.3 5-Flourouracil

5-Flourouracil is one of five drugs that are active in this disease. Other drugs given are Leucovorin, Irinotecan and Oxaliplatin along with two monoclonal antibodies (Kocáková,
2011). Statins are also useful. Since they shift stem cells to more differentiated cells by altering gene expression thereby enhancing the effect of 5-Fluorouracil (Kodach et al., 2011). 5-Flourouracil seems to be an effective drug if given in advanced stages of colorectal cancer (Jonker, Spithoff and Maroun, 2011). 5-Flourouracil is given in post operative patients with locally advanced colorectal cancer (Sauer et al., 2004). The polymorphism expressed in gene profile of patients being treated with 5-Flourouracil may be responsible for adverse effects that are seen during the therapy (Scalvini et al., 2012).

5-Flourouracil is a drug that is pyrimidine analogue which is used in the treatment of cancer (figure3). The hydrogen atom in position 5 of uracil is replaced by similarly sized atom of fluorine and the molecule was designed to occupy the active sites of enzyme, thus blocking metabolism in malignant cells (figure3). It causes irreversible inhibition of thymidylate synthase. It belongs to family of drugs called anti metabolites. 5-Flourouracil blocks pyrimidine synthesis by inhibiting the action of thymidylate synthase and thus inhibiting DNA replication. Thymidylate synthase methylates deoxyuridine monophosphate (dUMP) into thymidine monophosphate (dTMP). 5-Flourouracil causes decrease in dTMP, thus proliferating cells undergo thymineless death. 5-Flourouracil like other chemotherapeutic drugs effects rapidly dividing cells which require excessive use of nucleotide synthesis such as cancer cells.

The CD95/CD95 ligand system is seen to be involved in chemotherapeutic drug induced apoptosis. 5-Flourouracil induces apoptosis through the activation of CD95/CD95L system (Eichhorst, Müerköster, Weigand and Krammer, 2001).

1.4 Antibody

Bak belongs to Bcl-2 family of proteins which is characterized by the ability to modulate apoptosis under physiologic condition. The function of Bak is to increase apoptic cell death following appropriate signals thus working to encounter protection from apoptosis that is being offered by Bcl-2. The p53 and Bcl-2 family of proteins are involved in chemotherapy induced apoptosis in a cell type depended manner (Nita et al., 1998). In order to study the role played by p53 and Bcl-2 family of protein in 5-flourouracil induced apoptosis in HCT 116p53+/− human colon cancer cell line, 5-flourouracil with inhibitory concentration of 50% (IC50) was used to trigger apoptosis. This was further conformed by western blotting.
1.5 HCT116 p53 cell line

P53 is a tumor suppressor gene involved in DNA damage response. Colon cancer cells undergo mitochondrial mutations that are repaired by base excision repair (BER) mechanism. p53 plays a role in enhancing this BER mechanism by direct interaction with repair complex (Chen, Yu, Zhu and Lopez, 2006). 5-Flourouracil exposure to HCT116 p53+/- colorectal cell line with deficiency of mismatch repair characteristic resulted to decrease the number of viable cells through apoptosis.

2. Aim

The aim of this study was to observe the effectiveness of drug 5-Flourouracil on HCT116 p53 +/- colon cancer cells when used to treat colon cancer cells. The aim of this experimental study was also to investigate if the effect of 5-Fluorouracil is dependent on time or on concentration.

3. Materials and methods

3.1 Chemicals and Cell Culture

5-Flourouracil was purchased from Sigma Aldrich (St.Louis, MO) and product number of this drug was F6627 in powder form which was then solubilized in DMSO stock solution of drug. 10mg/ml was prepared and stored at -4°C. The XTT toxicology kit and McCoy’s cell culture medium were purchased from Sigma Aldrich and they were prepared following the protocol provided along with it.

The HCT116 P53 +/- colon cancer cells were obtained from American type culture collection (ATCC, Bethesda, MD;ATCC#CCL 247). These cells were cultured in 75ml flask in McCoy’s media which comprised of 10% fetal bovine serum (FBS), PEST (GIBCO Invitrogen) Carlsbad, CA, USA and L-Glutamine (GIBCO Invitrogen) Carlsbad, CA, USA (1.5mM). The flask containing cells and media were then kept in incubator with 95% air and 5% CO2 at 37°C. Trypsin triple express was used each time to harvest cells after 48 hours of incubation.
3.2 Experimental design

3.2.1 Concentration – Dependent Assay

After trypsinization, cells were counted by means of neaubeaur haemocytometer and approximately 30,000 cells were seeded per well in the 96 well plate. These cells were put into incubator and allowed to be attached to the surface of the wells, after being incubated for 24 hours, the medium was removed from all 96 wells and the cells that were attached to the surface of the wells were again re suspended with medium containing 5-Flourouracil drug at different concentration of 100µM, 75 µM, 50 µM and 25µM. All the cells in wells were treated except for the control cells and wells that were made blank by adding only medium into them with no cells. The control wells contain cells with media and DMSO. Total 60 out of 96 wells were seeded. From these 60 wells 6 wells were made as control and 6 wells were made as blank. For each drug concentration 12 wells were used out of 60 wells of 96 well plates. Other 36 wells of 96 well plates were filled with PBS solution. The 96 well plate was again incubated for another 24 hours, the medium was removed from the wells and new medium containing drug at different concentration of 100µM, 75µM, 50µM and 25µM was added and allowed to be incubated for another 48 hours. Two replicates for each drug concentration were prepared. Concentration dependent assay were repeated thrice.

3.2.2 Time Dependent Assay

After trypsinization the cells were counted by using Neaubeaur Haemocytometer and approximately 30,000 cells were seeded in four different 96-wells plates and were allowed to be incubated for 24 hours. Total 60 out of 96-wells were seeded. From these 60 wells 6 wells were made as control and 6 wells were made as blank. 48 wells were treated with IC50 value of the drug. Other 36 wells of 96 well plates were filled with PBS solution. After 24 hours medium was removed from all four plates and new medium was added to plates containing drug 5-Flourouracil of concentration 50µM. This concentration of drug was obtained from concentration dependent assay and was found to be IC-50 value of drug 5-Flourouracil for cell line being used in the experiment. Thus cells in the different 96-wells plates were treated with 5-Flourouracil at a concentration of 50µM and evaluated after 24 hours, 48hours, 72 hours and 96 hours. After
every 24 hours new drug containing medium was added to the plates. Time dependent assays were repeated thrice.

3.2.3 XTT Assay

XTT reagent comprises of (2, 3-bis [2-Methoxy-4-nitro-5 Sulfo-phenyl]-2H-tetrazotium-5-carboxyanilide salt). XTT reagent is in powder form. It was thus solubilized with 5ml of fresh media and the suspension was completely solubilized by keeping in warm water bath at 54ºC for few minutes. This XTT solution is light sensitive and thus stock solution was wrapped in aluminum foil paper and stored at -20ºC.

The purpose of using XTT method was used to estimate the number of cells viable in the wells by using measuring absorbance of wavelengths of 405nM and 595nM. For this purpose standardized micro plate readers using the excitation filter in Fluorostar (BMG Fluorostar micro plate reader) were used. These measurements were taken 4 hours after adding XTT to the wells each time and the procedure was managed to be done in darkness because of XTT being light sensitive. Thus after adding XTT to the wells, the 96 well plates were allowed to be incubated for at least 4 hours before measurements were done.

In concentration dependent assay cells after 48 hours of treatment were exposed to the XTT regent and XTT assay was performed. According to XTT assay the concentration of drug at which half of the cells were left viable was 50µM after 48 hours. This concentration was interpreted on plotting a graph and thus was marked as IC-50 values.

3.3 Western blotting

3.3.1 Protein Extraction Reagent

Freshly prepared RIPA Buffer- containing 50mM Tris (PH-8.0), Nacl (150mM),SDS (0.1%) and 1%Triton X 1% Sodium deoxycholate. BCA-Protein Assay Kit- purchased from Thermo Scientific (23225, pierce biotechnology) was used for protein measurement. Working reagent A and B was prepared from BCA protein Assay kit manual (thermo.com/pierce).
3.3.2 Protein Extraction

After trypsinization the cells were counted by using Neaubeaur Haemocytometer and approximately 774,000 cells were seeded per well in 3, 6-well plates. The loaded cells were allowed to be attached to the surface of wells then incubated for 24 hours. After 24 hours the medium was removed from all 6-well plates and incubated for another 24 hours. Drug treatment was done for all wells using IC50 concentration of drug 5-fluorouracil that is 50 µM except control wells to which only medium containing DMSO was added. After 24 hours of incubation the cells from one plate were removed using PBS and trypsin and the other 2, 6 well plates were once again treated and put into incubator for another 24 hours. In each 6-well plate 2 wells were kept as control containing cells with media and other 4 wells containing cells were treated with IC50 value of drug 5-fluorouracil. The cells removed were subjected to protein extraction by using RIPA buffer mixed with protease inhibitor cocktail tablet purchased from ROCHE Diagnostics GmbH. These extracted protein samples after being centrifuged at 13000rpm for 15minutes at 4ºC were then put in 4ºC refrigerator and the supernatant was discarded.

In the same way the remaining two, 6-well plates were also trypsinized after 48 hours and 72 hours and protein were extracted both from the control as well as treated wells and samples were stored in 4ºC refrigerator after being labeled as C24, T24, C48, T48, C72 and T72.

Now all the samples containing extracted proteins were measured using BCA proteins Assay kit.

3.3.4 Antibodies

Table 1: Following antibodies were used in western blotting

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bak</td>
<td>Rabbit polyclonal</td>
<td>Goat anti-rabbit</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Mouse monoclonal IgG antibody.</td>
<td>Goat anti- mouse IgG-AP conjugated.</td>
</tr>
</tbody>
</table>

Table 1 show the antibodies used in western blotting. Bak and β-Actin were purchased from Santa Cruz biotechnology. The purpose of doing western blotting was to analyze the expression of proteins. It was done according to Bio-Rad manual. 10-wells were made by using 1.5 mm
spacer comb to provide more volume (66 µL) per well. Protein volume was calculated to provide 50 µg as concentration for every protein sample. The protein whose expression is required to be analyzed was Bak whereas β-Actin was used as internal control in this study (table 1). The protein samples both from control and treated at 24 hour, 48 hours and 72 hours were loaded into gel mixed with sample buffer. Ladder of 250kDa size was also loaded on each ends of gels. Then Electrophoresis of gels was done using 200 volts for approximately 35 to 40 minutes. The gels were then transferred to the membranes using semi dry transfer apparatus and this was done for 30 minutes. The membranes were washed with washing buffer and blocked with nonfat milk for 15minutes. Then membranes were exposed to Bak and β-Actin being used as primary antibodies and left in cold room and 4 degree overnight on rocking machine. Then the membranes were washed with TBS/T and TBS and exposed to secondary anti bodies that matched with primary anti bodies and then after one hour membranes after being washed with TBS/T and TBS were exposed to BCIP tablet solution. In few minutes bands appeared.
4. Results

After incubating the HCT116 p53+-cells for 24 hours, the cells appeared as uniform monolayer of cells when viewed under the microscope. The cells were seen adherent to the surface of the cell culture flask. However after 48 hours of incubation more than 80% confluence was observed.

![Figure 4: shows the cells in the control wells in A at 24 hours of incubation and B at 48 hours of incubation thus showing that the number of cells increase with passage of time after incubation in the control well.](image)

The cell in control wells after 48 hours of incubation are more (figure 4B) as compared to the cells in control well after 24 hours of incubation (figure 4A). The control wells contained cells with media. This demonstrates that cells provided with growing media tend to multiply (figure 4B) with passage of time.

4.1 Concentration dependent assay

The HCT116 p53+-human colon cancer cell line was treated with drug 5-Fluorouracil at different concentrations of 25 μM, 50 μM, 75 μM and 100 μM and were then incubated for 48 hours. These treated cells in 96 well plates when viewed under the microscope showed considerable change when compared with the cells made as control.
Figure 5: shows the microscopic observation of cells treated with different concentration of drug that is 25 µM, 50 µM, 75 µM and 100 µM in figure A, B, C and D respectively. The cell number is more when treated with lowest concentration of drug 25 µM (figure 5A), cell concentration is less when treated with maximum concentration 100 µM (figure 5D).

The cells in the treated wells were seen to be damaged. 5-Flourouracil produced cytotoxic effect by damaging DNA which in turn affected cell proliferation and survival. The cytotoxic activity of drug on the cells increased gradually as the concentration of drug was increased. When cells were treated with different concentration of drug the cell viability was decreased with increase in concentration of the drug. The cell viability was more when treated least concentration of drug (figure 5A) then it decreased gradually when drug concentration was increased to 50 µM and 75 µM (figure 5B, 5C) and cell viability was seen least when treated with maximum concentration of drug 100 µM (figure 5D). Thus highest cytotoxicity of cells were seen in the wells treated with maximum concentration of drug that is 100 µM (figure 5D) as compared to the wells in
which cells were treated with lowest concentration of drug that is 25 µM (figure 5A). Thus as concentration of drug increased the cytotoxic effect of drug also increased.

4.2 XTT concentration dependent curve

The results from XTT concentration dependent assay were plotted on graph and IC-50 value that is the value at which half of the cells loose the viability was found to be 50 µM (figure 6). The graph demonstrates that as the concentration of drug increases, the cell viability continues to decrease (figure 6). Thus the cell viability was 63% when cells were treated with 25 µM concentration of drug and cell viability was 32% when treated with 100µM concentration of drug after 48hours of treatment. In second experimental trial cell viability was 66% when treated with 25 µM concentration of drug and 15% when treated with 100 µM concentration of drug when treated for 48hours. The concentration dependent experiment was repeated thrice. The values of cell viability with different concentration of drug were plotted on graph and demonstrated that as concentration of drug was increased the number of cells decreased (figure 6). This result was concluded by plotting the values on graph. In this case no control was used to compare the values rather values of cell viability with different concentration of drug were compared with each other.

Figure 6: Represents XTT Concentration dependent Graph
4.3 XTT time dependant curve

From this experimental trial it was observed that when colon cancer cells were treated with IC-50 value of this experiment that is 50 µM concentrations of 5-Flourouracil drug for different time intervals that is 24hour, 48hours, 72hours and 96hours the cell viability continued to decrease gradually with passage of time (figure7). Time dependent assay were done twice.

![XTT Time dependent Graph](image)

Figure 7: XTT Time dependent Graph

Table 2: The following table represents the concentration of protein extracted from human colon cells both control and treated for 24hour, 48hours and 72hours.

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Concentration (µg/ul) (24 hour)</th>
<th>Concentration (µg/ul) (48 hour)</th>
<th>Concentration (µg/ul) (72 hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Sample</td>
<td>1.2</td>
<td>1.89</td>
<td>5.14</td>
</tr>
<tr>
<td>Treated Sample-1</td>
<td>1.1</td>
<td>3.91</td>
<td>2.83</td>
</tr>
<tr>
<td>Treated Sample-2</td>
<td>1.4</td>
<td>2.9</td>
<td>1.5</td>
</tr>
</tbody>
</table>
4.4 Western blotting

In this experimental trial western blotting was done to see the expression of Bak proteins. β-Actin was used as internal control. Concentration of protein that was obtained from the samples both for control and treated at 24 hours, 48 hours and 72 hours were calculated (table 2) and then samples were loaded in the gel. Bands for β-Actin appeared to be uniform in all samples however it was weaker in the first control sample due to less amount of protein (figure 8). Bands for Bak were obtained in all samples at 24hours, 48hours and 72hours (figure 8). However for treated samples, bands for Bak become weaker with the passage of time (figure 8). Western blotting was done twice

![Western Blot](image)

Figure 8: Shows bands for β-Actin and Bak

Figure 8 expression of Bak antibody on human colon cancer cells after treatment with 5-Flourouracil. Western blot using β-Actin as internal control and Bak antibody. The figure shows expression of Bak decreases with passage of time after being treated (T24, T48 and T72)

Bands for Bak were when compared with β-Actin showed that band for Bak was strong in treated sample at 24hr then it became weaker in treated sample at 48hr and still weaker in treated sample at 72hr as compared to β-Actin (figure 9).
Figure 9: Diagrammatic comparison of expression of Bak and β-Actin antibodies.
5. Discussion

5-Flourouracil has been a chemotherapeutic drug of choice for many years for colorectal cancer. 5-Flourouracil has been in use for more than 30 years and its efficacy as a single chemotherapeutic agent has been considered to be less than 30% (Presant et al., 2000). Resistance to therapeutic efforts has been observed in the treatment of advanced colorectal cancer and thus continues to rely on the use of fluoropyrimidines (Huang, Horvath and Waxman, 2000). Research is being done to biomodulate 5-Flourouracil in order to improve the cytotoxic and therapeutic effects of this drug in the treatment of advanced disease (Malet-Martino and Martino, 2002). Apoptosis is end result of cells treated with chemotherapeutic agents (Nita et al., 1998). In chemotherapy induced apoptosis, p53 and Bcl2 family of proteins are involved (Nita et al., 1998). HCT 116 p53+/- human colon cancer cell line was predicted to have no resistant because of it being deficient in mismatch repair (Sergent et al., 2002). Thus exposure of HCT 116 p53+/- cell line to effective concentration of 5-Flourouracil resulted in reduction of viable cells through the induction of apoptosis. In order to determine 5-Flourouracil at inhibitory concentration of 50% (IC50) doses, was used to induce apoptosis (Nita et al., 1998). In this study IC50 value was found to be 50 µM. Bak antibody which is a member of Bcl2 family of proteins expression was analysed by western blotting. The mean IC50 value of 5-Flourouracil observed across National Cancer Institute NC160 panel of human tumor cell lines, was 17.6 µM with the range being 1-501 µM (Watters et al., 2004). 5-Flourouracil induced apoptosis demonstrated decreased expression of Bak without modulation of other Bcl2 family of proteins (Nita et al., 1998). In this study expression of Bak became weaker with passage of time in treated samples. It has been studied that interaction of p53 with Bak leads to oligomerization of Bak and thus releasing cytochrome C from mitochondria. This interaction results in loss of connection of Bak with antiapoptotic Bcl2 family. These interactions results in loss of connection of Bak with antiapoptic Bcl2 family. These interactions results in modulation of activity of death effect Bak (Leu et al., 2004).

6. Conclusion

This experimental study demonstrated 5-Flourouracil to be an effective drug hence being capable of producing cytotoxic effect when treated against HCT116 p53+/- human colon cancer cell line.
Concentration dependant assay revealed 50 µM concentration of drug to be effective in causing cell viability to decrease to 50% when used. Time dependant assays showed that this effective concentration of drug if used over a period of time leads to considerable decrease in cell viability with passage of time. Western blotting technique showed the activity of Bak protein in the process of apoptosis. Thus it can be concluded that 5-Flourouracil appears to be effective cytotoxic drug if used alone in treating human colon cancer cells.
7. References


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Appendix

- **6-well plate**: Plate having approximately 9.5cm² and it is used for culturing the mammalian cells in 6 well-plates for getting the protein by protein extraction.
- **96-well plate**: Plate having approximately 0.32cm² and it is used for culturing mammalian cells.
- **APS**: Ammonium Per sulfate- it is used for catalyze the polymerization during making gel.
- **BCIP/NBT**: 5-bromo 4- chloro 3- indolphosphate p-toluidine salt/Nitro blue tetrazolium chloride.
- **DMSO**: Dimethyl Sulfoxide- It is used in the preparation of stock solution of drug.
- **Sample Buffer**: 1.25 ml of 0.5M Tris HCL of PH-6.8 were mixed with 2.5ml glycerol, 2.0ml 10%SDS and mixed with 3.55ml deionized water, 0.2ml (0.5%) Bromophenol blue.
- **SDS**: Sodium dodecyl sulfate polyacrylamide.
- **TEMED**: N, N, N, N.tetramethylethylendiamine-Used for polymerization during gel preparation.
- **Washing Buffer**: 24.2 g Tris Base mixed with 80g NaCl and pH were adjusted to 7.6 and made up to 1000ml.
- **XTT**: it is Cell Viability Assay Kit and it is important for determination of viable cells.

Preparation of chemicals

- **1.5 M Tris-HCL, pH 8.8**
  27.23g Tris Base, 80 ml dH₂O
  The pH should be 8.8 by 6N HCl. Total volume should be 150 ml with dH₂O and store at 4°C.

- **0.5 M Tris-HCl, pH 6.8**
6 g Tris Base, 60 ml deionized water
The pH should be 6.8 by 6N HCl. Total volume should be 100 ml dH$_2$O and store at 4°C.

- **Sample Buffer**
  
  1.25 ml 0.5 M Tris-HCl, pH 6.8
  2.0 ml 10 % SDS
  3.55 ml dH$_2$O
  2.5 ml glycerol
  0.2 ml 0.5 % Bromophenol blue

- **10x Electrode (Running) Buffer, pH 8.3**
  
  144.0 g Glycine
  30.3 g Tris-Base
  10.0 g SDS
  The total volume should be 1,000 ml with dH$_2$O.

It should be store at 4°C. If precipitation starts, then it should be warm to room temperature before use.

- **10 % SDS**

  Dissolve 10g SDS in 90 ml of water with gentle shaking and stirring and bring to 100 ml with dH$_2$O

- **10 % APS (fresh daily)**

  100 mg ammonium per sulfate and it should be dissolve in 1ml of dH$_2$O.

- **10x Washing Buffer (TBS)**

  80 g NaCl
  24.2 g Tris-Base
  Adjust pH to 7.6 and bring to 1000 ml.

- **TBS/T**

  1xTBS + 0.1% Tween

- **Transfer Buffer**

  25 mM Tris, pH 8.3
192 mM Glycine
20 % MeOH (Methanol)
0, 25 – 0, 1 % SDS

- **Gel casting**

Because of the size of protein Bcl-2, a resolving gel percentage 12 % was suitable for the full separation of protein in the Western Blot. The gel was mixed according to Table 1. Assemble the gel casting equipment and then put the water for checking the leakage.

<table>
<thead>
<tr>
<th>Percentages of gel that is used in WB</th>
<th>DDI H2O (ml)</th>
<th>30 % Acrylamide (ml)</th>
<th>*Gel Buffer</th>
<th>10 % SDS (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stacking Gel 4%</td>
<td>6.1</td>
<td>1.3</td>
<td>2.5</td>
<td>0.1</td>
</tr>
<tr>
<td>Resolving gel 12%</td>
<td>3.4</td>
<td>4</td>
<td>2.5</td>
<td>0.1</td>
</tr>
</tbody>
</table>

For resolving gel buffer- 1.5 M Tris-HCL at pH-8.8
For stacking gel buffer- 0.5 M Tris-HCL at pH 6.8
For resolving gel:

50µl 10% APS and 5µl TEMED
For stacking gel:

50µl 10 % APS AND 10µl TEMED

**Protein extraction standard curve**
Figure 10: Shows standard curve for protein extraction