

Efficacy of Capecitabine and 5- Fluorouracil (5-FU) on the human breast cancer cell line (MCF7) – effect of concentration

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Abstract

Background: 5-Fluorouracil and capecitabine are antimetabolite drugs which are used for colorectal cancer and metastatic breast cancer which is resistant to first line chemotherapy drugs. Capecitabine mimics continuous infusion 5-fluorouracil and generates 5-FU preferentially at the tumor site through exploitation of high intratumoral thymidine phosphorylase concentrations. According to previous clinical studies, capecitabine has fewer side effects, less complications, better efficacy and is better tolerated than 5-FU. The present study investigates the evaluation of the efficacy of 5-FU and capecitabine on MCF-7 breast cancer cell line.

Methods: The method employed was MTT assay to find out the half maximal inhibitory concentration (IC₅₀) of both drugs. The IC₅₀ was obtained by plotting the concentration (µg/ml) versus the percentage of inhibition of each drug.

Results: The MCF7 cell line had no response to capecitabine within 24 hours. We could get results after 48 and 72 hours, due to the low potency of capecitabine. According to the results, after 48 hours treatment of MCF7 cell line with capecitabine, in the concentration of 1147.9 µg/ml 50% of cells have been inhibited. After 72 hours treatment of the cells with capecitabine, at the concentration of 921 µg/ml 50% of cells have been inhibited. 5-fluorouracil was effective

on the MCF7 cell line after 24 and 48 hours. After 24 hours treatment of MCF7 cells with 5-Fu, in the concentration of 1.3 µg/ml 50% of cells have been inhibited, and after 48 hours, in the concentration of 0.38 µg/ml 50% of cells have been inhibited.

Conclusion: The results showed that the IC₅₀ of 5-FU was comparably lower than the IC₅₀ of capecitabine. In fact, 5-FU demonstrated better efficacy on the MCF-7 cell line as compared to capecitabine. In other words, 5-FU is 3021 folds more potent than capecitabine.

Key Words: 5-fluorouracil, capecitabine, MTT assay, MCF7 cell line.

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Introduction

5-fluorouracil is an antimetabolite which is present as IV injection and topical dosage forms. 5-Fu exists as 500 mg injection (50mg/ml, 10 ml single dose vials) for intravenous injection. 5-fluorouracil is usually used for the treatment of slowly growing solid tumors, such as colorectal, breast, ovarian, pancreatic, and gastric carcinoma. It is also useful in the treatment of superficial basal cell carcinoma when applied topically. After administration, 5-fu enters the cells through a carrier mediated transport system. Inside the cell, it will be converted to 5-fluorodeoxyuridine monophosphate (5-FdUMP). 5-FdUMP competes with deoxyuridine monophosphate for thymidylate synthase. Thymidylate synthase is an enzyme which converts deoxyuridine

monophosphate to deoxythymidine monophosphate (dTMP). As a result of interference of 5-fu with the function of thymidylate synthase, the formation of dTMP will be interrupted, and thus, DNA synthesis will be stopped.⁽¹⁾

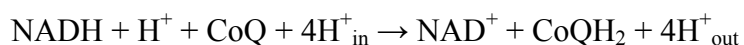
Capecitabine is an oral fluoropyrimidinecarbamate which is used for the treatment of colorectal cancer and metastatic breast cancer which is resistant to first line chemotherapy drugs.⁽¹⁾Capecitabine is the prodrug of 5-fu. After it is taken orally, it undergoes a series of enzymatic reactions which leads to its hydrolysis to 5-fu.⁽²⁾Capecitabine converts to 5-fu in the body through a cascade of three enzymes, including “carboxylesterase”, “cytidine (Cyd) deaminase”, and “thymidine phosphorylase”.⁽³⁾ After ingestion of capecitabine, it passes intact through the intestinal tract into the portal vein, and goes to the liver. In the liver, it will be converted to “5'-deoxy-5-fluorocytidine (5'-dFCyd)” by the enzyme carboxylase. After that, it will be converted to “5'-deoxy-5-fluorouridine (5'-dFUrd)” by the enzyme Cyddeaminase which is present in the liver and tumor tissues. Finally, it will be converted to 5-fu by the enzyme thymidine phosphorylase, which is present in the tumor tissue. Capecitabine is much safer than 5-fu, and has less side effects.⁽⁴⁻⁸⁾

The MTT Cell Proliferation Assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. The MTT Reagent yields low background absorbance values in the absence of cells. For each cell type the linear relationship between cell number and signal produced is established, thus allowing an accurate quantification of changes in the rate of cell proliferation. This assay utilizes 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), a water soluble yellow dye that is readily taken up by viable cells and reduced by the action of mitochondrial dehydrogenases. The reduction product is a water insoluble blue formazan that must then be dissolved for colorimetric

measurement. Formazan can be used to detect the presence of very small numbers of living cells. Metabolically inactive cells, such as erythrocytes do not produce significant numbers of formazan. Conversely, the amount of Formozan produced per cell in a given time is dependent on the metabolic activity of that cell; activated lymphocytes generate up to ten times as much formazan as resting cells. An advantage of MTT assay is that tetrazolium-cleaving enzymes are not present in serum. A disadvantage is that this is a destructive technique in that the cells cannot subsequently be used for any other assay. However, the culture medium can be saved for other assays if required.⁽⁹⁾

In this method, after adding MTT reagent, if there is any live cell in the culture, the tetrazolium salt will be converted to Formosan. Live cells produce energy by mitochondria. NADH dehydrogenase, (also referred to as NADH:ubiquinonereductase or Complex I) is an enzyme located in the inner mitochondrial membrane that catalyzes the transfer of electrons from NADH to coenzyme Q (CoQ). CoQ is an oil-soluble, vitamin-like substance is present in most eukaryotic cells, primarily in the mitochondria. It is a component of the electron transport chain and participates in aerobic cellular respiration, generating energy in the form of ATP.

The reaction of NADH dehydrogenase is:



In this process, the complex translocates four protons across the inner membrane per molecule of oxidized NADH, helping to build the electrochemical potential used to produce ATP.⁽¹⁰⁾

The MTT reagent (tetrazolium salt) will take up the protons produced by the mitochondria dehydrogenases. Through this mechanism, tetrazolium will be converted to formazan, and the

cell culture will change color from colorless to purple color. This is the process which happens when the culture contains live cells. On the other hand, when the cells are dead, there will be no more dehydrogenases activity in their mitochondria, and thus, no more protons will be produced in these cells. As a result, in the absence of proton, tetrazolium cannot be converted to formazan, and the cells will not change color. Thus, after adding tetrazolium salt, if the cell culture has changed color, we can detect the presence of live cells. However, if the cell culture has not change color, we can detect the presence of dead cells.

The IC₅₀ is the concentration of an inhibitor where the response (or binding) is reduced by half. The half maximal inhibitory concentration (IC₅₀) is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. This quantitative measure indicates how much of a particular drug or other substance (inhibitor) is needed to inhibit a given biological process (or component of a process, i.e. an enzyme, cell, cell receptor or microorganism) by half. In other words, it is the half maximal (50%) inhibitory concentration (IC) of a substance (50% IC, or IC₅₀). In this assay, through plotting the graph, we will determine the IC₅₀ of two drugs.⁽¹¹⁾

The objectives of this study are to determine antiproliferative effect of capecitabine and 5-FU on breast cancer cell line, to compare the efficacy of capecitabine and 5-fluorouracil on the human breast cancer cell line (MCF7), to determine IC₅₀ of these two compounds, and to compare the cytotoxicity of these two compounds on breast cancer cell line.

Materials and methods

Materials

Capecitabine were kindly provided by OsvahPharmaceutical Company respectively (Tehran, Iran). 5 FU was provided from SigmaAldrich (Germany). Human breast cancer cell lines MCF-7 were kindly provided from the Department of Pharmacy (Faculty of Medicine, University of Malaya). Media-RPMI 1640 from SgmaAldrich (Germany), Fetal Bovine Serum (FBS) from GIBCO (Life technologies, USA0, The MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazoliumbromide) substance and all the other reagents and substances were obtained from SigmaAldrich (Germany). The stock cultures were grown in 25 cm² culture flasks and all experiments were carried out in 96 microtitre plates (NUNC, Denmark). The experiment was conducted under a laminar air flow cabinet (Labconco, purifier class II biosafety cabinet) provided by University Malaya, Malaysia.

Method

Plating of cells and treatment of cells with the drugs: Confluent (80-90 %) cells were taken out from incubator (WTC binder) for plating. Cells were viewed under an inverted microscope (Olympus CK40) to check the percentage of confluence and to confirm the absence of bacterial and fungal contaminations. The media from the flask containing confluent cells was removed and the cells rinsed with 3 ml of fetal bovine serum (FBS). Then 1.5 ml of trypsin was added into 25 cm² flask and incubated at 37 C in a humidified incubator under 95 % O₂ and 5 % CO₂ for 5 min to detach the cells. After cells were detached, 3 ml of media containing RPMI and 5 % FBS was added to the dispersed cell layer to inactivate the trypsin. The cells were centrifuged for 5

minutes at 4000 rpm, the supernatant was removed and RPMI with 5% FBS was added and shaken thoroughly. In order to count the number of MCF7 cells in the media, 10 μ l of the media was taken and mixed with 10 μ l of trypan blue. 10 μ l from the mixture was taken and placed on a neobar tube. The neobar slide was placed under a microscope and the number of cells present in the 4 squares of the neobar slide was counted. The number of cells existing in 1 ml of the media was calculated through the following calculation:

$$\frac{\text{number of cells counted}}{4} \times 2 \times 10000 \quad (\text{eq. 1})$$

The total number of cells counted on the neobar slide was 700, thus,

$$700/4 \times 2 \times 10000 = 35 \times 10^5 \text{ Cells/ml} \quad (\text{eq. 2})$$

The required number of cells in each well is 7000. In order to calculate the amount of the media to be transferred in each well, we used the following equation:

$$\frac{1000 \times 7000}{35 \times 10^5} = 0.2 \mu\text{ln 1 well} \quad (\text{eq. 3})$$

Thus, 0.2 μ l of media is transferred to each well. If we assume the total number of wells in each plate is 100, then totally 20 μ l of media is required for whole the plate. 100 μ l of media should be in each well. Thus, we need totally 10000 μ l. Thus, we added 9980 μ l of RPMI to the media. The media was transferred to the boat, and the cells were transferred to the plate using eight channel pipette. The plate was placed in the incubator for 24 hours. After 24 hours, the cells were treated with different concentrations of the drugs, 5-flurouracil and capecitabine. The concentrations of 5-florouracil are 15, 4.5, 1.35, 0.4, 0.12 μ g/ml, and the concentrations of capecitabine are 1600, 1400, 1200, 1000, 800, 600, 400 μ g/ml. The plate was designed as follow:

Two plates for 5-Fu and two plates for capecitabine have been designed. The plates for 5-Fu were designed in such a way that wells A1 to A5 contain the drug for highest concentration, and from the wells A to E the concentration of drugs decrease gradually. The wells E1 to E5 contain the drug with lowest concentration. The wells F1 to F5 contain the media only. The plate was designed according to the figure 1.

The plates for capecitabine were designed in such a way that the wells A1-A5 contain the drug with highest concentration. From the wells A to G the concentration of drug decreases gradually. The wells G1 to G5 contain the drug with lowest concentration. The wells H1 to H5 contain the media only. The plate was designed according to the figure 2.

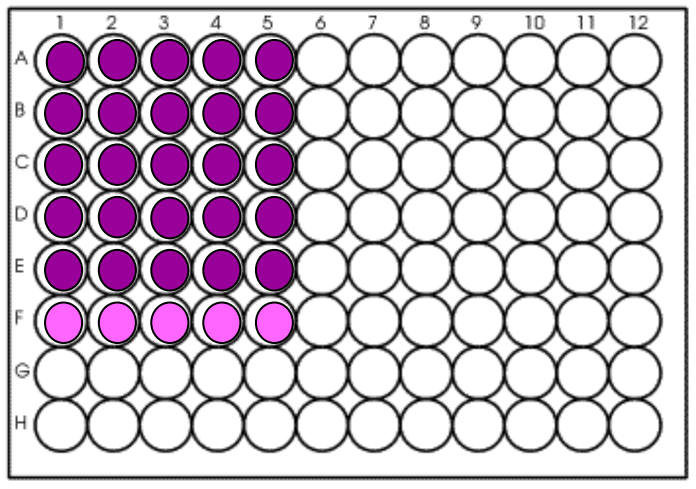
After treating the cells with the drugs, the plates were placed in the incubator under 95 % O₂ and 5 % CO₂. One of the plates containing 5-Fu was placed in the incubator for 24 hours and the other plate of 5-Fu was placed in the incubator for 48 hours. One of the plates containing capecitabine was placed in the incubator for 48 hours, and the other one for 72 hours.

MTT Assay: After removing the plates from the incubator, 10µl MTT to each well, using eight channel pipettes. Then, the plates were placed in a shaker incubator for 4 hours. After removing the plates from incubator, the content of the plates were through away and 10 µl DMSO was added to each well using eight channel pipette. After that, the plates were placed in the microplate reader (Cole SPC, 1986). After getting the absorbance value from the microplate reader, we calculated the percentage of inhibition using the following equation:

$$\frac{(\text{absorbance of control} - \text{absorbance of sample})}{\text{absorbance of control}} \times 100$$

$$\text{Inhibition\%} = \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100 \quad (\text{eq. 4})$$

The experiment has been repeated for 3times. The data have been entered to Microsoft excel, and the graphs have been attained.





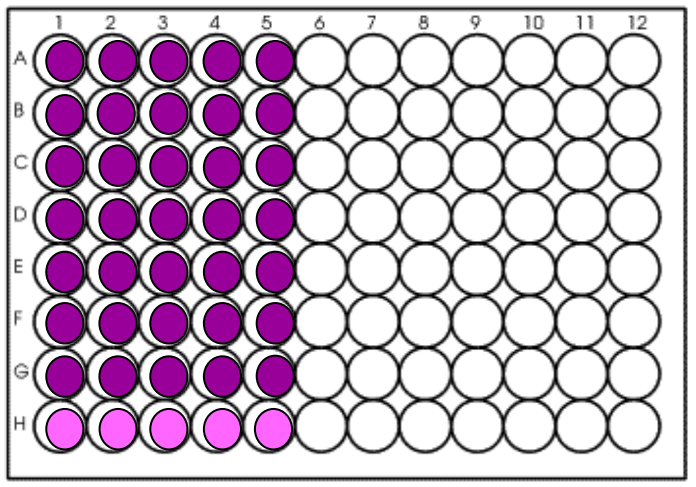
- 5-Fu 
- Media 

Figure 1: the plate treated with 5-Fu.





- Capecitabine 
- Media 

Figure 2: the plate treated with capecitabine

Results

1. Effect of capecitabine within 48 hours

MCF7 breast cancer cells were precultured in 96-well microplates (7000 cells per well) for 24 h and then incubated with capecitabine (1600-400 $\mu\text{g/ml}$) for 48 h. The cytotoxicity of capecitabine in MCF7 cells was evaluated based on its effect on cell growth by using the MTT assay (Fig.3). The cytotoxicity of capecitabine was dose-dependent, the maximum cell death seen at concentration of 1600 $\mu\text{g/ml}$. The medium inhibitory concentration (IC_{50}) of the drug on MCF7 cells was 1147.91 $\mu\text{g/ml}$.

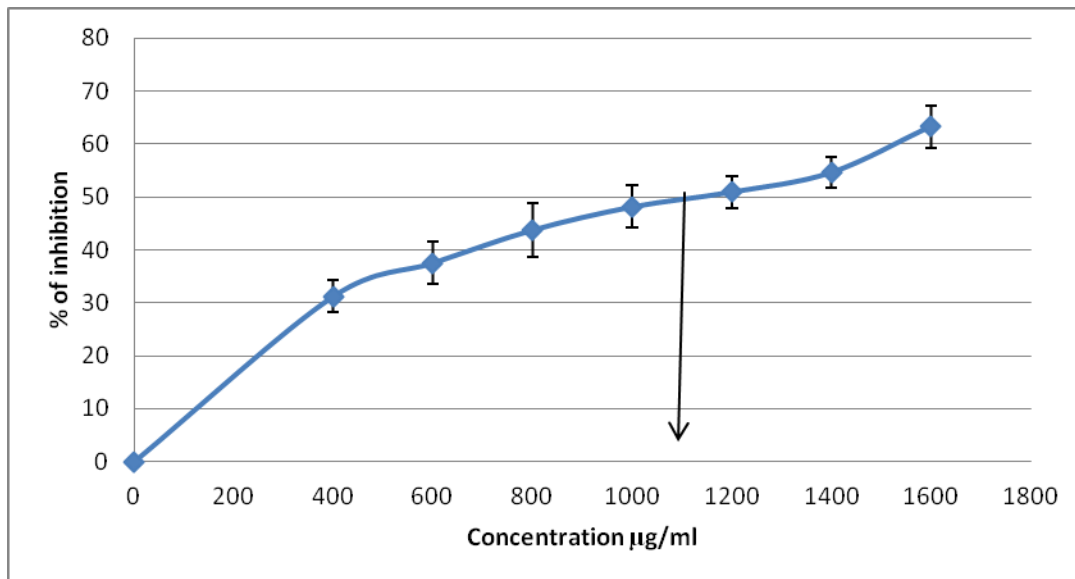


Figure 3: Effect of *capecitabine* on MCF7 cell viability after 48 hours. Data are presented as mean S.D. (n=3). $\text{IC}_{50} = 1147.91 \pm 14 \mu\text{g/ml}$.

2. Effect of 5-flurouracil within 48 hours

MCF7 breast cancer cells were precultured in 96-well microplates (7000 cells per well) for 24 h and then incubated with 5-flurouracil (15-0.12 $\mu\text{g/ml}$) for 48 h. The cytotoxicity of 5-Fu in MCF7 cells was evaluated based on its effect on cell growth by using the MTT assay (Fig. 4). The cytotoxicity of 5-Fu was dose-dependent, the maximum cell death seen at concentration of 15 $\mu\text{g/ml}$. The medium inhibitory concentration (IC_{50}) of the drug on MCF7 cells was 0.38 $\mu\text{g/ml}$.

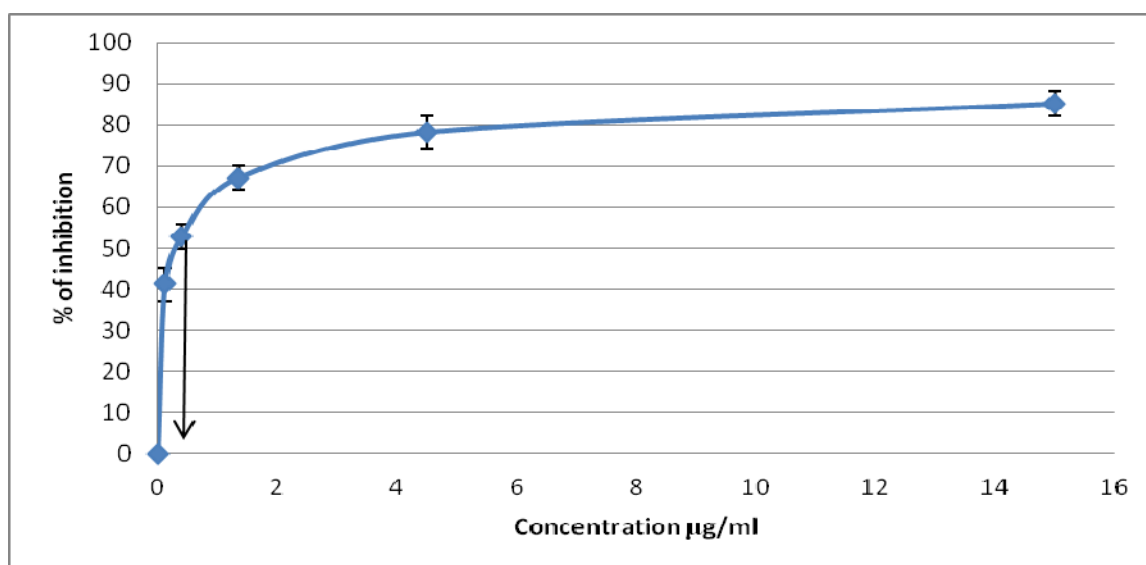


Figure 4: Effect of 5-fu on MCF7 cell viability after 48 hours. Data are presented as mean \pm S.D. (n=3). $\text{IC}_{50} = 0.38 \pm 0.02 \mu\text{g/ml}$

3. Effect of Capecitabine within 72 hours

MCF7 breast cancer cells were precultured in 96-well microplates (7000 cells per well) for 24 h and then incubated with capecitabine (1600-400 $\mu\text{g/ml}$) for 72 h. The cytotoxicity of capecitabine in MCF7 cells was evaluated based on its effect on cell growth by using the MTT assay (Fig. 5). The cytotoxicity of capecitabine was dose-dependent, the maximum cell death seen at concentration of 1600 $\mu\text{g/ml}$. The medium inhibitory concentration (IC_{50}) of the drug on MCF7 cells was 921.1 $\mu\text{g/ml}$.

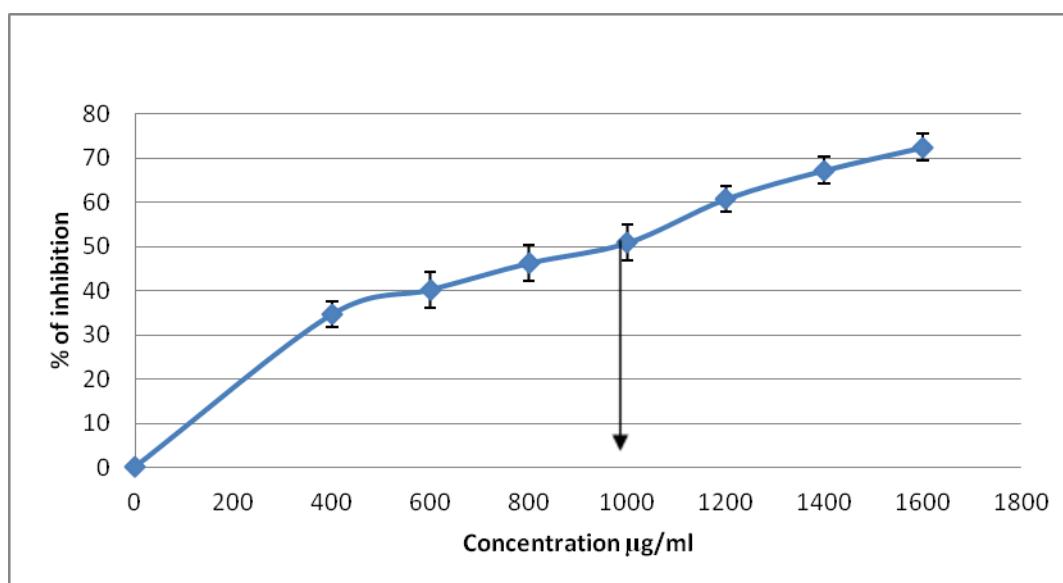


Figure 5: Effect of *capecitabine* on MCF7 cell viability after 72 hours. Data are presented as mean \pm S.D. (n=3). $\text{IC}_{50} = 921.1 \pm 14 \mu\text{g/ml}$.

4. Effect of 5-flurouracil within 24 hours

MCF7 breast cancer cells were precultured in 96-well microplates (7000 cells per well) for 24 h and then incubated with 5-flurouracil (15-0.12 $\mu\text{g/ml}$) for 48 h. The cytotoxicity of 5-Fu in MCF7 cells was evaluated based on its effect on cell growth by using the MTT assay (Fig. 6). The cytotoxicity of 5-Fu was dose-dependent, the maximum cell death seen at concentration of 15 $\mu\text{g/ml}$. The medium inhibitory concentration (IC_{50}) of the drug on MCF7 cells was 1.3 $\mu\text{g/ml}$.

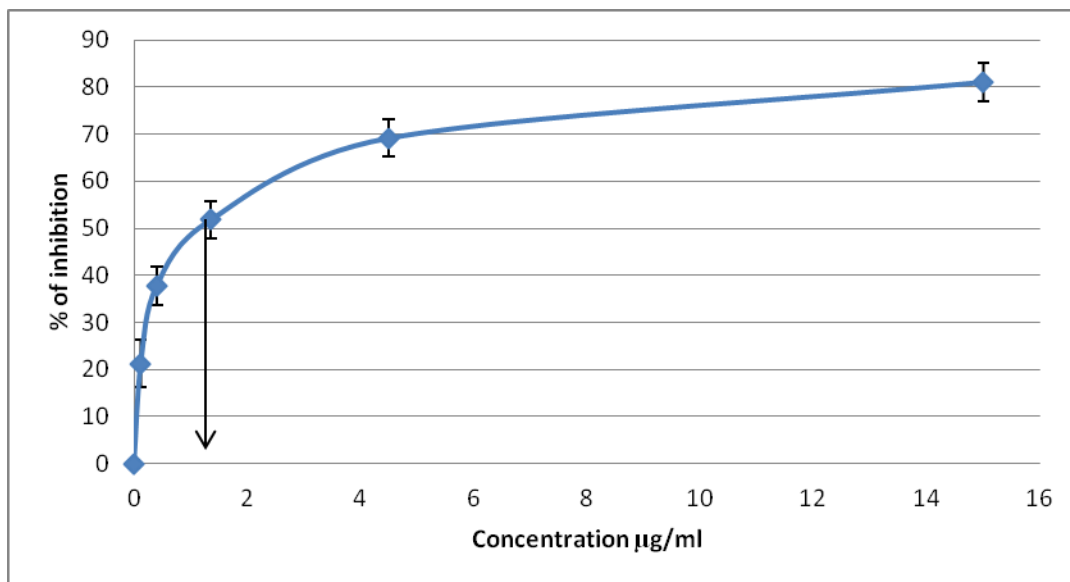


Figure 6: Effect of 5-*fu* on MCF7 cell viability after 24 hours. Data are presented as mean

\pm S.D. (n=3). $\text{IC}_{50} = 1.3 \pm 0.02 \mu\text{g/ml}$.

Discussion

In this study, two drugs with different concentrations have been applied on the MCF7 breast cancer cell line. Previous studies show that 5-fu is more toxic and has a lower IC50. Capecitabine is the prodrug of 5-fu, thus, lower toxicity and higher IC50 is expected.

The MCF7 cell line had no response to capecitabine within 24 hours. The results were attained after 48 and 72 hours, due to the low potency of capecitabine. According to the results, after 48 hours treatment of MCF7 cell line with capecitabine, in the concentration of 1147.9 $\mu\text{g/ml}$ 50% of cells have been inhibited. After 72 hours treatment of the cells with capecitabine, at the concentration of 921 $\mu\text{g/ml}$ 50% of cells have been inhibited.

5-fluorouracil was effective on the MCF7 cell line after 24 and 48 hours. After 24 hours treatment of MCF7 cells with 5-Fu, in the concentration of 1.3 $\mu\text{g/ml}$ 50% of cells have been inhibited, and after 48 hours, in the concentration of 0.38 $\mu\text{g/ml}$ 50% of cells have been inhibited.

A study done by Hector H.V, et al, on transcriptional profiling of MCF7 breast cancer cells in response to 5-fluorouracil, shows that the IC50 of 5-fu on MCF7 cell line after 48 hours is 10 μM , which is equal to 1.3 $\mu\text{g/ml}$. The difference between the result of Hector's study and the result of this study can be due to the difference in the materials used, such as the media which is DMEM in Hector's study and RPMI in this study. There are also some differences in the amount of materials used, such as the percentage of FBS which is 10% in Hector's study and 5% in this study. The other difference, is the number of cells seeded in each well, which is 4 \times 10⁴ in Hector's study and 7 \times 10³ in this study. (Vargas H.H, et al, 2006)

Another study shows that the IC₅₀ of capecitabine on MCF7 breast cancer cell line after 48 hours was 1120 µg/ml, while this study determines the IC₅₀ of capecitabine to be 1147.9 µg/ml after 48 hours.(12)

Although there are some differences between the results of this study and the two other studies, all of them indicate that capecitabine has a much higher IC₅₀ than 5-Fu. The difference in the IC₅₀ of these two drugs is due to the difference in their mechanism of action. In fact, capecitabine should be converted 5-Fu in the body by the enzymes which exist inside the body, such as carboxyl esterase which exists in the liver, cytidine deaminase which exists in both liver and cancerous tissue, and thymidine phosphorylase which exists in the cancerous tissue (tumor site). In the MCF7 breast cancer cell line, the transformation of capecitabine to 5-Fu will not take place completely. As a result, when the cells are exposed to capecitabine, a smaller number of them will be inhibited, while when 5-Fu is added to the cell line, a much greater number of cells will be inhibited.

Conclusion

The results of this study shows that the IC₅₀ of 5-Fu is much lower than that of capecitabine, thus, the toxicity and inhibitory effect of 5-Fu is much greater than capecitabine. In fact, 5-Fu is 3021 folds more potent than capecitabine. So, it can be concluded that the greater inhibitory effect of 5-FU on MCF-7 is due to its higher toxic potential on the cancerous cell. Despite the advantages of 5-FU, since the efficacy of 5-FU is limited due to its rapid dehydration into dehydro-50 flurouracil, capecitabine is more commonly administered as an anticancer drug.

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