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DETERMINATION AND COMPARISON OF IC_{50} VALUES OF ANTICANCER DRUGS DOXORUBICIN, DOCETAXEL ON HUMAN OVARIAN AND COLON CELL LINES BY MTT ASSAY

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ABSTRACT

Cancer occurs when the cells acquire the property of immortality and keeps on dividing without a halt. The body keeps a check on the normal cell division rate but sometimes this control is lost and it leads to the formation of tumors, which may be benign or malignant. For cancer studies a part of the tumor is taken from the origin location and cultured till it becomes a stable cell line to be used for in-vitro experimentation. The cytotoxic effect of Doxorubicin and Docetaxel are observed on Human Ovarian and Colon cell lines namely PA1 and SW620. Doxorubucin is classified as an "anthracycline" drugs and is one of the most widely used anticancer drugs. Doxorubicin damages DNA by intercalation of the anthracycline portion, metal ion chelation, or by generation of free radicals.[1] Docetaxel (Taxotere) is of the chemotherapy drug class; taxane, and is a semisynthetic analogue of paclitaxel (Taxol) Docetaxel binds to microtubules reversibly with high affinity. This binding stabilises microtubules and prevents depolymerisation from calcium ions, decreased temperature and dilution.[2] The effectiveness of drugs was observed at different drug concentrations by MTT, (3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) assay. Transforms, plotting and analysis of the experimental data was done by Graph Pad Prism version 4.03.

KEYWORDS : Doxorubicin, Docetaxel, Anticancer, MTT

I. INTRODUCTION

Cancerous cells always remain in the dividing phase, they become confluent. Confluency refers to the coverage of proliferation that the cells are allowed over or throughout the culture medium. For example, a confluency of 40-60% will mean that there may be little or no restrictions to the growth of cells in/or the medium and they can be assumed to be in a growth phase. [3]

Therefore, it is necessary to maintain these cells regularly. The cancerous cells consume the media very rapidly and require renewal of media. Some cell lines require renewal every day. Since, the cells do not die out; their numbers has to be reduced due to limited space in the culture flask. Some cells are frozen and preserved as a "back up" in case, and the culture gets contaminated.

II. MATERIALS AND METHODS

Maintenance of Cancerous Cell Lines

Following methods were used for the manufacture of cell lines:

- Revivals of cells from freeze downs.
- Renewal of media
- Sub culturing and Trypsinization
- Preparation of freeze downs

Revival of Cells from Freeze Downs

Materials : Cryopreserved vial, Water bath, Centrifuge, DMEM , FBS, CO2

Incubator

1. Required vial was removed from the freezer and thawed to room temperature.
2. The contents of the vial were transferred to 15 ml centrifuge tube containing 5-6 ml of media and FCS followed by a centrifugation at 1400 rpm for 4 mins.
3. The supernatant was discarded and pellet was re-suspended in the required volume of media containing FCS.
4. The suspension was transferred to T-25 culture flask and kept for incubation at 37°C in a CO2 incubator.

Renewal of Media

When the cancer cells are cultured, with the pace of time, all the essential components of the media are consumed and the media becomes little acidic due to the production of metabolites by the cells. "Phenol Red Indicator" which turns yellow in acidic medium indicates this condition.

Procedure of renewal of media

1. Exhausted medium was taken out from the culture with the help of pipette and discarded it in the disposal flask.
2. Fresh media was poured in the culture. Volume of the media depends on the type of cell line and the culture flask in which it was plated.
3. While pouring media from the pipette to the culture, it

was advised to avoid formation of air bubble in pipette, otherwise it may cause contamination.

Sub-culturing and Trypsinization

When the cancer cells become confluent, there is lack of space in the culture flask and therefore, they start to peel-off. Sometimes, they grow in multi layer, making it difficult to handle. If the culture were left in this condition, after some time all cells would die.

Since all cells are adherent to the culture flask, they are removed by the addition of trypsin-EDTA, which digests the protein responsible for adhesion. This process is called trypsinization. The transferring of cells from one culture to another is called subculturing or passaging.

Materials :- Trypsin-EDTA, PBS, DMEM supplemented with FBS,15ml, culture flask, CO2 incubator, 50 ml centrifuge tube(falcon)

Procedure for trypsinization

1. The exhausted media was taken from the culture and discarded.
2. 4-5 ml of trypsin-EDTA solution was added to the culture and the flask was moved gently for about 30-40 seconds so that trypsin-EDTA spreads all over the cells.
3. Trypsin-EDTA was removed, leaving few drops in the flask.
4. The flask was rotated up and down, so that the Trypsin-EDTA flows over all the cells.
5. When the cells started to detach from the surface, the flask was gently tapped from the sides such that all the cells in the solution come in suspension.
6. 10-20 ml of fresh media containing serum was added immediately after the formation of suspension. This stops the action of Trypsin-EDTA. If the cells were left in trypsin-EDTA for long, the cells would come under the stress.
7. The solution of the media and Trypsin-EDTA was removed, leaving few drops in the flask.
8. Fresh media was added in the flask and the culture is incubated in CO2 incubator at 37°C

Procedure for Sub-culturing/passaging

1. The confluent flask was selected for sub-culturing.
2. Adherent cells were detached by trpsinization.
3. A small volume was taken out into a fresh culture flask containing the fresh media along with serum, thus expanding the cell number.
4. After each trypsinization there occurs an increment of one in the passage number.
5. Flasks were stored in CO2 incubator.

In-vitro cytotoxicity screening using MTT assay

MTT assay is a standard colorimetric assay for measuring cellular proliferation. MTT is a tetrazolium salt, which is yellow in color and is photosensitive. MTT, **(3-(4,5- Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)** is taken by the living cells and reduced by a mitochondrial dehydrogenase enzyme to a purple formazan product that is impermeable to the cell membrane. So the formazan product accumulates in the living cells.solublization with the solvents like DMSO leads to the liberation of product which can readily be detected.thus the conversion is directly related to the cell viability. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death of cells can be deduced, through the production of a dose-response curve. [4]

Procedure for screening

Materials required

Hemocytometer, cover slip, 15 ml centrifuge tube, centrifuge, vortex, 96 well ELISA plate, CO2 incubator, experimental cell lines (SW620 and PA1 cell lines).

Chemicals required

Trypsin-EDTA, DMEM+FCS, Doxorubicin, MTT, DMSO

Plating of cells

1. The exhausted media was discarded and 3-4 ml of Trypsin-EDTA was added.
2. After few minutes the Trypsin-EDTA solution was aspirated and 4-5 ml of the media with the serum was added.
3. A clean hemocytometer was taken along with the glass cover slip.
4. Cell suspension was vortexed and 10µl of the suspension was placed on the hemocytometer.
5. The slide was observed under the phase contrast microscope.
6. The cells were counted in the four corner squares.
7. The hemocytometer and the cover slip were cleaned.

8. The cells were plated in the microtitre plate using a multi channel pipette.
9. The plate was kept in CO2 incubator for overnight incubation.

Counting of cells and calculations

Let the no of cells in 16 squares = 67

Total no of cells per ml = 67 X 104 = 0.67 million cells/ ml

For plating 3000 cells per well,

Total no of cells to be plated would be = 3000x 110

= 0.33 million cells/ well

Each well contains media = 180 µl

110 wells will contain the amount of media = 110 x 180 µl

= 19.8 ml cells are present in 1ml of the media

0.67 million cells will require the suspension volume= $(0.33 \times 10^6) \div (0.67 \times 10^6) = 492$ µL

Addition of drug

1. Dilutions of the drug Doxorubicin and Docetaxel were prepared in serum free DMEM from the stock solution of 2.05mg/ml in the concentration range of 100µM, 10µM, 1µM and 0.1µM
2. Cells were treated with the respective dilution in 1:10 ratio so as to make the final concentrations 1/10th of the initial prepared.
3. The plates were kept for 72hours/3 days incubation in a CO2 incubator at 37°C.

Assay termination using MTT

1 After the 72 hours/3 days incubation the reactions in the plate were terminated by addition of 20 µl of MTT in to each well of the micro titer plate and kept for 3 hours incubation in a CO2 incubator at 37° C
2. After 3 hours the entire media was aspirated out and discarded without disturbing the formazan complex.
3. The formazan complex was dissolved in 150µl of Dimethyl Sulfoxide.
4. The ELISA plate was read at 540nm and the percent inhibition in the cell growth was determined.
5. IC50 was estimated by using the graph pad prism software and data was analyzed.

III. RESULTS AND DISCUSSION

MTT cytotoxicity assay was performed on two cell lines namely PA1 and SW620. The data was obtained after the plates were read on Anthos™ ELISA plate reader. The obtained data was later used for the calculation of IC50(EC) value (the half maximal inhibitory concentration i.e the concentration of the Inhibitor required for the 50% inhibition of the target of the drug that was used on the given cell lines. The software used for transforms, plotting and analysis was Graph Pad Prism version 4.03.

Doxorubicin Conc. (µM)	Percentage Inhibition (SW620)	PI (PA1)
0.01	11.50	10.27
0.10	26.73	19.74
1.00	71.98	75.58
10.00	85.60	85.06

	SW620	PI (PA1)
Sigmoidal dose-response (variable slope)		
Best-fit values		
BOTTOM	0.0	0.0
TOP	100.0	100.0
LOGEC50	-0.4940	-0.4314
HILLSLOPE	0.6445	0.8368
EC50	0.3206	0.3703
Std. Error		
LOGEC50	0.1061	0.1665
HILLSLOPE	0.09409	0.2312
95% Confidence Intervals		
LOGEC50	-0.9507 to -0.03735	-1.148 to 0.2851
HILLSLOPE	0.2397 to 1.049	-0.1562 to 1.832
EC50	0.1120 to 0.9176	0.07114 to 1.928
Goodness of Fit		
Degrees of Freedom	2	2
R²	0.9851	0.9598
Absolute Sum of Squares	55.06	175.2
Sy.x	5.247	9.359
Constraints		
BOTTOM	BOTTOM = 0.0	BOTTOM = 0.0
TOP	TOP = 100.0	TOP = 100.0
Data		
Number of X values	4	4
Number of Y replicates	1	1
Total number of values	4	4
Number of missing values	0	0

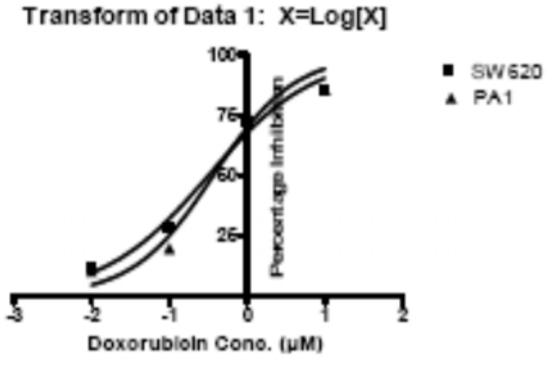


Fig .1 IC50 value of Doxorubicin in PA1 cell line is 0.3206 µM and for SW620 it isin 0.3703 µM

Docetaxel Conc. (µM)	PI (SW620)	PI (PA1)
0.001	10.61	12.43
0.010	22.51	23.41
0.100	51.84	56.92

	PI (SW620)	PI (PA1)
Sigmoidal dose-response (variable slope)		
Best-fit values		
BOTTOM	0.0	0.0
TOP	100.0	100.0
LOGEC50	-1.042	-1.184
HILLSLOPE	0.5195	0.5500
EC50	0.09082	0.06543
Std. Error		
LOGEC50	0.07926	0.1221
HILLSLOPE	0.05595	0.09778
95% Confidence Intervals		
LOGEC50	-2.049 to -0.03481	-2.736 to 0.3676
HILLSLOPE	-0.1914 to 1.230	-0.6924 to 1.792
EC50	0.008936 to 0.9230	0.001836 to 2.331
Goodness of Fit		
Degrees of Freedom	1	1
R²	0.9930	0.9811
Absolute Sum of Squares	6.331	20.28
Sy.x	2.516	4.503
Constraints		
BOTTOM	BOTTOM = 0.0	BOTTOM = 0.0
TOP	TOP = 100.0	TOP = 100.0
Data		
Number of X values	3	3
Number of Y replicates	1	1
Total number of values	3	3
Number of missing values	0	0

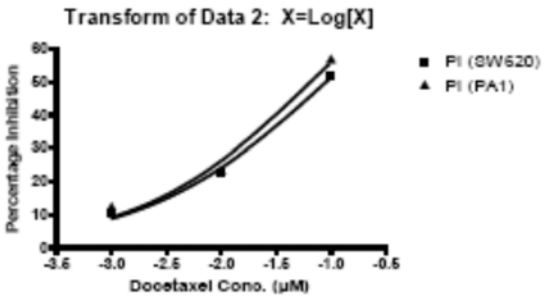
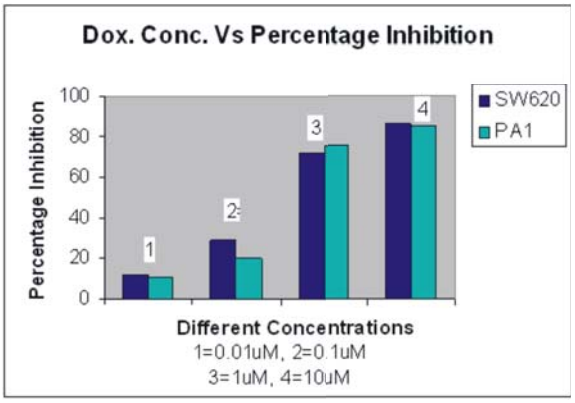
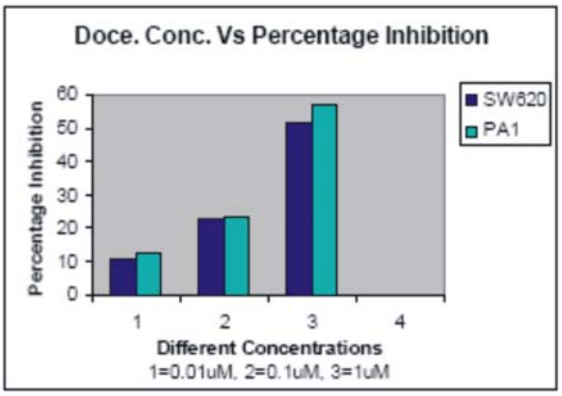


Fig .2 IC50 value of Docetaxel for SW620 cell line is 0.09082 and for Pa1cell line it's found to be 0.06543



Graph 1 : Doxorubicin Concentration Vs Percentage Inhibition in SW620 and PA1



Graph 2 : Docetaxel concentration versus Percentage inhibition in SW620 and PA1 cells

IV. CONCLUSION

On the basis of the observations and software analysis it can be concluded that:

- Doxorubicin is a very potent anti cancer drug with an appreciable cytotoxicity on PA1 (cancerous ovarian cell line) and SW6320 (colon cancer line). IC50 value of Doxorubicin on PA1 cell line was found to be 0.3206 µM and for SW620 it was 0.3703 µM. Doxorubicin is also found to be cytotoxic to normal cells so the drug can have appreciable side effects.
- It was observed that the maximum apoptosis was seen with 10µM Doxorubicin concentration as compared to lower concentrations. Thus, the higher the concentration of drug, higher will be the rate of apoptosis of the cells. It means that higher concentration of the drug will lead to the death of a large number of cells. But the amount of apoptosis is not linearly dependent on the drug concentration.
- Doxorubicin shows similar amount of Percentage Inhibition for both SW620 and PA1 cell lines whereas the Percentage Inhibition effected by Docetaxel is different on PA1 and SW620.
- At lower concentrations Dox. Is more effective on SW620 cells than on PA1 but at higher conc.'s the inhibition is similar.
- Percentage Inhibition of Docetaxel on SW620 just lags behind its effect on PA1 throughout the concentration range.

- It is also concluded that a higher conc. of Docetaxel could have been used to get better statistical analysis.

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