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Curcumin Co-Treatment Sensitizes Multi-Drug Resistant Ht29 Colon Cancer Cell Line

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Abstract

Colorectal cancer has been confirmed to be the third most dreadful cancer across the world. The latest reports show that the colorectal cancer is increasing in India at a high rate due to food habits, particularly consuming the foods with high fat content. Chemotherapy has been considered as a potential treatment in the control of a wide range of cancers recently, such as gastrointestinal cancers and so on. The scientists are looking to come up with new drugs to treat cancer by regulating the carcinogenesis with minimal toxicity.

Curcumin is a phytochemical extracted from turmeric. It is highly effective product of the rhizomes of *Curcuma longa* L. (Zingiberaceae). It is also considered as a valuable drug for chemotherapeutic treatments in cancer. In case of animal studies, it has been clearly mentioned that curcumin controls carcinogenesis in various other organs as well as colon. Curcumin exhibits the capability to act as anti-mutagenic, anti-inflammatory drug as well. It has also been confirmed that the P-gp expression regulation is carried out by curcumin by inhibiting the COX-2 expression. Also, it has been clearly stated that curcumin down-regulates NF-kB pathway in various cell lines like Colo 205 colon cancer cell line etc. Multi Drug Resistance (MDR) plays a key role in case of the cancer cells to exhibit the resistance against the cytotoxicity of various chemotherapeutic drugs. This type of characteristics in cancer cells is developed due to low levels of the chemotherapeutic drug accumulated inside the cells during repeated exposure to the drug, showing the over-expression of P-glycoprotein (MDR-1). P-glycoprotein, a transporter protein, allows the cells to expel the wide range of chemotherapeutic drugs. The latest reports show that COX-2 expression and P-gp expression possess strong correlation with each other.

In this research study, we generated the Multi-Drug Resistant HT29 Human colon cancer cells by treating the cells with increasing concentrations of a chemotherapeutic drug namely Doxorubicin Hydrochloride (DOX) and further we estimated the intracellular drug concentration in treated live as well as dead cells with the help of Doxorubicin Accumulation Assay. Also, the cell morphology change was studied in HT29 cells after every 24 hours of the DOX treatment separately as well as curcumin co-treatment (DOX+Curcumin). The curcumin co-treatment was carried out to observe the effect of curcumin on multi-drug resistant HT29 colon cancer cells and further study can be extended to investigate the various inflammatory genes controlled by NFkB at mRNA and protein level as well as the levels of cytokines which may get down-regulated by treatment. This study will help to completely understand the mechanism of reducing the effect of Multidrug Resistance (a unique property of cancer) by curcumin in case of colon cancer.

Keywords: HT29 cells; Multi-drug resistance (MDR); Doxorubicin Hydrochloride (DOX); P-glycoprotein (P-gp); Colorectal cancer (CRC); Cyclooxygenase (COX)

Introduction

Colorectal cancer (CRC) is reported to be one of the most familiar causes of death in men and women, and has been ranked as the third most common deadly cancers worldwide which includes the estimation of approximately 6, 08, 000 mortality cases contributing nearly about 8% of all cancer related deaths. This is the malignant tumor which gets developed in the gastrointestinal track of the body. In India, the colon cancer and rectal cancer incidence rates in men are 4.4 and 4.1 per 1, 00,000 and in case of women, it has been estimated to be 3.9 per 1, 00,000 according to the latest rates. Recently a study was conducted in case of colorectal cancer in which, a total of 224 colorectal tumour cases were evaluated by the Cancer Genome Atlas Network, the pattern of genomic alterations in colon and rectal tissues studied intensely ,was found to be related to a greater extent, without taking anatomic location and origin into consideration. In this case, the researchers came to the conclusion that the colon as well as rectum tumours can be grouped together. In this study, a set of 24 genes were found to be mutated in a several number of cases. New genes such as SOX-9, FAM123B / WTX, ERBB2, and IGF2 were identified in addition to other genes found already in previous research (for example APC, ARIDIA, TP53, KRAS, and PIK3CA). These genes were found to be highly involved in regulation of cell proliferation and can therefore it has been proved that these genes can act as potential therapeutic drug targets. The various risk factors in case of colorectal cancer include two broadly classified factors such as; genetic and environmental or lifestyle-related factors. Most of the cases in colorectal cancer are sporadic, although genetic factors increase the risk at a high rate.

The occurrence of colorectal cancer is still at an increasing rate in spite of our enhanced understanding of the pathogenesis of this disease, as well as various improved screening strategies for this malignancy have been established so far. It has been shown that almost 50% of the patients with colorectal cancer develop recurrent disease, which indicates that the prevailing treatment regimens are not in a position to control this deadly disease and improved as well as advanced therapies are needed at a higher rate in this case. Most of the tumours

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activate the transcription factor nuclear factor KB (NFKB), whereas natural chemo preventive agents suppress these transcription factors, exposing a strong link between tumor biology and the anticancer effects of various natural compounds. Colon cancer occurs as a result of stepwise accumulation of genetic alterations in various genes leading to enhanced genomic instability. These genetic changes have direct impact on metastasis associated genes, oncogenes and tumor suppressor genes. Natural active compounds have a key role to play in regulating the genes involved in colon cancer, thus revealing the possible ways to control such a dreadful disease.

The HT29 colon cancer cell line (adherent cell line) at the very first was derived by Fogh and Trempe [1] from a Caucasian primary tumour localized in the colon of a 44 year old Caucasian female. The HT29 cells possess some useful characteristics as in normal tissue, for example, hormone receptors etc. Also, in the state of several different culture conditions, HT29 cells can differentiate in polarized monolayers of mucus-secreting and/or absorptive cells. This cell line secretes various peptides and several factors which act as stimulators for the proliferation of fibroblasts.

It has been clearly confirmed that consuming fruits and vegetables is very helpful in subsiding the risk of malignancy in tumours according to various recent epidemiological studies. Thus, several dietary polyphenolic phytochemicals that has the capability to control carcinogenesis and inhibiting the proliferation of colon cancer cells, have been taken for the process of clinical investigation. Curcumin, a phytochemical (a phenolic compound), is the yellow compound extracted from *Curcuma longa* L. (Zingiberaceae), it is a commonly known dietary product and is currently under research as a highly valuable drug for chemotherapeutic treatments in cancer. In case of animal studies, curcumin controls carcinogenesis and has been confirmed to possess the unique ability to act as antitumor, antioxidant, anti-arthritic, antiamyloid anti-ischemic in nature. Due to the potential anticancer effects of curcumin, apoptosis gets induced in cancer cells without showing any cytotoxic effects on healthy cells. Curcumin also shows the property to act as anti-mutagenic, hypoglycaemic, anti-inflammatory compound. Curcumin inhibits the activity of the transcription factor NF-κB, which in turn is linked to a significant number of inflammatory diseases such as cancer. Curcumin influences the expression of various genes like, metallothionein genes, tubulin genes, p^{53} etc., involved in colon carcinogenesis. Curcumin also plays an important role to express some of its therapeutic activities against the diseases like cardiovascular, loss of bone and muscle, depression and neuropathic pain etc. The P-gp inhibition by curcumin using irinotecan as P-gp substrate was recently studied with the help of in situ cancerous colonic single pass.

Perfusion method in rats: Irinotecan acts as a potential anticancer drug Exhibiting pharma coresistance. The P-gp expression regulation shown by Curcumin by inhibiting the COX-2 expression has also been reported. Multi Drug Resistance is the one of the most common characteristics of colon cancer cell line which gets developed inside the cancer cells by repeated exposure of a pharmacological drug. Due to low levels of the chemotherapeutic drug found inside the cells after continuous treatment, the cells show resistance to any cytotoxic effect of the respective drug. This causes the over-expression of P-glycoprotein (MDR-1) i.e. the drug efflux pump gets activated with the help of ATP hydrolysis. P-glycoprotein (ABC transporter) helps to efflux the range of compounds, chemotherapeutic drugs out of the cells so that there may be no effect of the drug on the cancer cell metabolism. The inhibition of P-gp can enhance the cancer treatment. There are a number of different mechanisms studied so far, which contribute effectively towards the development of MDR phenotype in cancer cells. These mechanisms include the specific target change of the particular drug, the reduced uptake ad increased efflux of the drug, the subsided capacity to undergo apoptosis, enhanced DNA damage repairment process etc. The recent reports have shown that COX-2 expression exhibits strong correlation with P-gp expression. It has also been reported that MDR-1 gene expression can be compared to NFkB in case of leukaemia K562 cell lines and hepatocellular carcinoma Hep-G2 cell lines. Thus, curcumin down-regulates NF-kB pathway in cell lines like Colo 205 colon cancer cell lines as reported recently.

Review of Literature

Colorectal cancer (CRC) is ranked among the most common cancers worldwide and one of the potential causes of death in men and women [2]. Although, the pathogenesis of this disease has been understood to a greater extent till date and as well as improved screening strategies has been developed for this malignancy, still its occurrence is rising at a high rate. The recurrent cases of the disease have been confirmed in about 50% of the patients having colon cancer, which indicates that currently available treatments to control the colon cancer are not highly efficient and there is the necessity of some enhanced therapies [3].

HT29 Human colon cancer cells are normally sensitive to the chemotherapeutic drugs such as 5-fluorouracil and oxaliplatin, these first line drugs are currently used in the treatment of colorectal cancer [4]. The colon cancer cells are considered to be feasible in-vitro model in order to study various parameters like absorption, transport, and secretion by intestinal cells and support a favourable experimental system for studying differentiation of epithelial cells. They form non-polarized multi-layers, results in undifferentiated phenotype [4]. However, their morphology can be changed in a better way by modifying the various culture conditions or treatment with various inducers to express several variations in the pathways of enterocyte differentiation [5-7]. The parental colon cancer HT-29 cell line usually consists of undifferentiated cells and less number of differentiated cells (3%-5% of total cells). Various subpopulations of absorptive HT-29 cell have been established so far, which has helped the researchers to extend the research in colon cancer .

Multidrug resistance (MDR) is the ability of cancer cells that are exposed to a given drug, with which these cells develop the different characteristics to resist the cytotoxic activity of a broad range of drugs, which are unrelated structurally and functionally. Although a number of factors usually contribute to MDR, the most important one among them is the over-expression of a plasma membrane ATPase called P-glycoprotein (P-gp) [8]. This enzyme increases the outward transport of active drugs and decrease their intracellular concentration and thus suppressing their cytotoxic efficacy [8].

Cyclooxygenase (COX) enzyme, also known as prostaglandin end peroxide H synthase (PGHS) is the important enzyme that accelerates the conversion of arachidonic acid to prostaglandins and eicosanoids. These Enzymes belong to a family of two isozymes i.e. COX-1 and COX-2 [9]. COX-1 regulates the homeostasis of various physiological functions and shows its presence in most of the cells [9]. COX-2 is considered to be inducible isoform of PGHS. It plays a key role by intervening in prostaglandin synthesis during inflammation and shows selective over-expression in many cancers [10-12]. The over-expression of COX-2 blocks the apoptosis process as well as an increased metastatic potential and neo-angiogenesis [13,14]. It has been reported that single nucleotide polymorphisms (SNPs) in the COX-2 modulates the enzyme function and elevates the risk of colorectal adenoma [15].

A strong link between tumours and the anti-cancer effects of various natural compounds has been reported which shows the possible ray of hope in colon cancer research. The important regulators of the host immune and inflammatory response regulators belong to NF-κB. Further, apoptosis of cells caused by DNA damage or cytokine treatment are regulated by NF-κB [16]. Tumours activate the transcription factor nuclear factor KB (NF-KB); while as many natural chemo-preventive agents supress it [17]. The chemotherapeutic agents, pro-inflammatory cytokines and radiation therapy induce cell apoptosis, but stimulate transcription factor NF-kB [16] leading to the chemo-resistance and radio-resistance of tumour cells [19]. It has been reported that drugs damage the cancer cell DNA, which then activates ATM kinase. The ATM kinase then activates the NF-κB essential modifier (NEMO), a component of the IKK complex, which in turn induces nuclear translocation of p^{65}/p^{50} transcription factor complex [20,21]. In tumour cells, when NF-kB is inhibited, cell cycle arrest occurs, thereby blocking cell proliferation and leading to apoptosis [22].

Plant polyphenols curb the signalling pathways that are responsible for development of resistance to chemotherapeutic drugs, thus play a vital role in reducing tumours. Curcumin, a naturally occurring yellow pigment of the spice turmeric, is proved to be a potent inhibitor of NF-kB activation [17,23]. Curcumin has been reported to have anti-tumor effects in a number of solid tumours and increase the sensitivity towards chemotherapy in drug-resistant cancer cells [17,22,24]. Curcumin has been found to inhibit the in-vitro colon cancer cell proliferation by entering into the G2/M phase [23]. Curcumin also affects NF-kB–regulated gene products such as caspase-3, -8, -9, PARP, Bax) (apoptosis), anti-apoptosis (Bcl-xL) and proliferation (cyclin D1) thereby, subsides cell proliferation and reduces the 5-FU-induced apoptosis in CRC cells [23].

Materials and Methods

Materials Used

The following important chemicals as well as other materials were used in this project phase.

Antibiotic/Antimycotic solution, Doxorubicin Hydrochloride (DOX), Curcumin, Dimethyl sulfoxide (DMSO), Disodium Hydrogen phosphate, Ethylene diamine tetra acetate (EDTA),Ethanol, Fetal Bovine Serum (FBS), Hydrochloric acid, Pottasium Chloride, Pottasium Dihydrogen phosphate, Sodium Chloride, O—phosphoric acid, Trypan blue dye, Cell culture dishes (100mm), Fluorescence plates (96 well), Centrifuge tubes (15mL, 50mL), Micro-centrifuge tubes (2mL, 1.5mL). These chemicals and other consumables were purchased from Himedia laboratories Mumbai, India. HT29 colon cancer cell line was purchased from National Centre for Cell Science (NCCS) Pune, India.

McCoy's 5A Medium:

McCoy's 5A Medium (500ml) consists of following composition:

1. McCoy's 5A medium powder - 6gm
2. Sodium bicarbonate - 1.1gm

McCoy's Medium was purchased from Himedia laboratories Mumbai, India as well as this medium was prepared by dissolving the above chemicals in 450ml of autoclaved Milli-Q water. The pH was checked using pH meter. The medium was then filtered using membrane filtration unit driven by vacuum pump through 0.22µm membrane filter paper. It was then transferred to an autoclaved bottle and stored at 4°C. The complete growth medium was prepared by adding 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution to the previously prepared McCoy's medium, as required for cell culture maintenance

Reagent Preparation:

a) 1X Phosphate Buffer Saline (PBS) Solution (1L)

1. Sodium chloride - 8gm
2. Potassium chloride - 0.2gm
3. Disodium hydrogen phosphate - 1.44gm
4. Potassium dihydrogen phosphate - 0.24gm

The above chemicals were dissolved in 800ml of distilled water and the pH was adjusted to 7.4. The volume was made up to 1L using distilled water and sterilized by autoclave.

a) 1X Trypsin-EDTA Solution (50ml)

1. Trypsin (0.25%) - 125mg
2. 0.53mM EDTA - 9.86mg

The above composition was prepared in 30ml of sterile 1X PBS solution and then made up to 50ml with autoclaved water. It was sterilized through 0.22µm syringe filter.

b) Lysis Mixture(50ml)

The lysis solution was prepared by adding 15mL of 1N HCl to 25mL ethanol and the volume was made up to 50mL using distilled water.

c) Trypan Blue Solution (0.4%)

The 0.4% trypan blue solution was prepared by adding 40mg of Trypan blue powder to 10ml 1X PBS solution.

d) Cryomedium (10ml)

The cryomedium was prepared by adding 5% of DMSO to 95% of Complete Growth Medium and stored at -20°C.

e) Bradford's Reagent preparation (250 ml)

Bradford's reagent was prepared by dissolving 25mg of Comassie Brilliant Blue G-250 dye in 12.5ml of ethanol. After dissolving completely, 25ml of O-phosphoric acid was added to it and 150 ml of distilled water as also added to it. The final volume was made up to 250ml using distilled water. The solution was stored in an amber bottle at room temperature.

Methods used

1. **Work plan**: The work plan for the phase of this project is sequenced below in a stepwise manner.

(Resistance Induction)

a) Generating MDR HT29 cells by treating the colon cancer HT29 cells with increasing Doxorubicin Hydrochloride concentration continuously for 7 days and check the chemo resistance by Doxorubicin Hydrochloride Accumulation Assay.

(Curcumin Co-treatment/Sensitization Check)

b) Treating HT29 cells with two different concentrations of Curcumin along with DOX treatment for 7 days continuously and check the

effect of curcumin on chemo resistance developed by DOX during co-treatment, with the help of fluorimetry..

Cell Culture

Human colon cancer cell line i.e. HT-29 was purchased from National Centre for Cell Sciences (NCCS), Pune, India. These cells were further Cultured in McCoy's 5A medium which was supplemented with 10% Fetal Bovine Serum and 1x antibiotic/antimycotic solution, and the cells after transferring them into the flasks, were placed for culture maintenance in 5% CO2 incubator set at 37°C. HT29 colon cancer cells were passaged continuously until high passage number was achieved and the cells were also freezed accordingly for future use.

Cell Line Revival

a) The cryopreserved vial containing freezed HT29 cells was thawed before use for maintaining the cultures as well as for the drug treatment process. The following steps were carried out in a sequential manner for the revival of HT29 cell line.

b) The frozen cryovial was taken out from liquid nitrogen and thawed quickly under slow stream of tap water at room temperature.

c) Then the cryovial volume was transferred to a centrifuge tube and the tube was centrifuged at 1500rpm for 5mins.

d) The supernatant formed was discarded completely in order to make sure that DMSO is completely removed from the pellet.

e) 1 ml of complete growth medium was added to the tube and the pellet was mixed completely and transferred to a culture flask.

f) The appropriate volume of the medium was added to the flask and incubated at 37°C and checked regularly for culturing purpose.

Passaging and Cryopreservation:

Passaging

The process of Passaging was carried out to maintain HT29 cell line. The cells were sub-cultured when the confluency reached to 70-80%. The confluency was checked with the help of inverted microscope. In this case, the old medium was removed from the flask and the flask was washed with sterile 1XPBS solution. The PBS was discarded and (1.5-2)ml of trypsin-EDTA solution was added to the same flask. The flask was then incubated at 37°C for 1 minute. The cells were checked under microscope for complete detachment. The trypsin solution was discarded and the flask was again incubated at 37°C for 5 minutes. After 5 minutes the complete growth medium was added to the flask and all the cells were collected at one point in the flask. The whole cell suspension was distributed uniformly to other fresh flasks accordingly and all the flasks were incubated at 37°C for further process.

Cryopreservation

Cryopreservation of HT29 cells was done in order to preserve the cells for future use. DMSO was used as a cryo-protectant for freezing HT29 cells when needed. HT29 cells usually having 80% confluency were cryopreserved. The old medium was discarded from the flask and the flask was washed with 1x PBS solution to avoid any dead cells. 2ml of trypsin was added to the flask and incubated for 1 minute at 37°C. Trypsin is discarded and again the flask was kept at 37°C for 5 minutes. The appropriate volume of medium was added to the cells and collected. The whole cell suspension was transferred to a centrifuge tube and centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded and the pellet was re-suspended in 1 ml of freezing medium containing 95% complete growth medium and 5% DMSO. The whole volume was transferred to the cryovial and the cryovial was placed at 4°C for 1-2 hours then at -20°C for 2-4 hours and then the vial was shifted to -80°C for overnight. In the morning on next day, the cryovial was taken out of the -80°C slot and immediately shifted to liquid nitrogen tank. The cryovials were labelled with cell line name, passage number and date before preservation.

Doxorubicin Resistance Generation

The two HT29 cell culture flasks were used for the DOX treatment. One normally cultured HT29 cell culture flask was treated with increasing concentrations of Doxorubicin Hydrochloride drug continuously for 7 days to check the resistance developed in the cells and the other flask was treated with constant concentration of DOX daily. This whole process was carried out with the help of two methods as short term treatment as well as for long term treatment.

Method 1:

a. 7 Days Treatment (Short term treatment): HT29 cells were continuously treated with DOX after every 24 hours for 7 days term ,with increasing concentrations of Doxorubicin drug (0.23 μM -1.61 μM).This treatment process was repeated several times to ensure high efficiency as well as to obtain an adequate amount of cells for Doxorubicin accumulation assay.

Method 2:

b. Continuous Treatment (Long term treatment): In this process, the HT29 cell culture flask was continuously treated with 68nM DOX after every 24 hours to generate the DOX resistant HT29 cells and were passaged when the confluency reaches to (80-90)%.The cells were passaged up to several passage number and have to be continued till it reaches to 20-25 passages to allow the cells to obtain the resistance against Doxorubicin.

Trypan blue Assay

The number of dead Cells was counted with the help of Trypan blue assay after every 24 hours during 7 days treatment process. The old medium was aspirated out of the treated flask in a 15 ml centrifuge tube regularly till the end of the treatment and was centrifuged at 1500 rpm for 5 minutes. The supernatant obtained, was discarded and the dead cell pellet was dissolved in 1ml of 1X PBS solution. 10μl of the dissolved sample was shifted separately in a fresh 1.5 ml eppendorf tube and 10μl of Tryphan blue dye was added and mixed properly to it. 10μl from this final volume was placed onto space provided for sample in the Haemocytometer apparatus. The sample after mounting was completely covered with coverslip to make it stable over the haemocytometer and enable it to get observed under the microscope without any spill action or any other disturbance. The dead cells stained blue due to penetrance of dye through the cell membrane were counted in all the 4 squares observed at 10x power under inverted microscope.

Doxorubicin Accumulation Assay

In case of 7 days DOX treated flask, at the end of the treatment on the next day after 24 hours, the medium containing the dead cells from the treated flask was aspirated out and transferred to a 15 ml falcon tube. The entire cells were washed twice with 1x PBS solution. The falcon tube containing dead cells was subjected to centrifugation at 1500rpm for 5 minutes. The supernatant was discarded and the appropriate volume of lysis solution was added to the pellet and the whole volume

was transferred to a fresh 1.5 ml micro tube. The cells present in the flask at the end of the 7 days DOX treatment after 1x PBS wash, were scrapped with the help of a cell scrapper and transferred into a fresh microtube after adding appropriate volume of lysis solution to the cells in the flask. Also, the first day treated (0.23μM DOX) HT29 cell flask was used for comparison purpose and the normal HT29 cell culture flask was used as control flask in this case. The microtube were labelled as 7 days treated live cells ,7 days treated dead cells, first day treated live cells, firstday treated dead cells and control cells accordingly. The cell samples were subjected to sonication process at 2 (10 seconds) bursts to lyse the cells for determining the DOX concentration inside the treated live as well as dead cells. The net volume of 200μl of each cell lysate sample after sonication process was transferred to the 96-well white opaque fluorescence plate along with the control (lysis solution) to measure the fluorescence. The fluorescence was read with the help of fluorimeter at 485 nm as excitation wavelength and 538 nm as emission wavelength. The DOX concentration was measured by plotting the graph in between the concentration and the fluorescence values obtained. This measurement is based on the calibration curve drawn for 5 different concentration of Doxorubicin hydrochloride in the range of 0.2nM/ml-7nM/ml.50μl volume of each cell lysate was used for protein estimation with the help of Bradford's assay. The formula used to determine the concentration of the unknown sample is cited below:

Concentration of unknown sample = (OD of sample/OD of standard)*concentration of standard/ (volume of sample)

The accumulation of drug per milligram of protein in the cell lysate was normalised with the help of following formula:

$$\text{DOX accumulation (nmol/mg)} = \frac{\text{DOX concentration (nmol/ml)}}{\text{Protein concentration (mg/ml)}}$$

In order to achieve high accuracy in the experiment, the treatment was repeated several times and also the cells were maintained in maintenance medium (complete growth medium containing 34nM/L DOX) if needed. The maintenance medium was regularly changed after every 24 hours in the 7 days DOX treated flask and incubated at 37°C till the further process. Along with 7 days treatment, continuous treatment was also started in order to maintain the cells till 20-25 passages continuously. In the meantime, Doxorubicin fluorescence spectrum was carried out as triplicates for five different concentrations (0.2-7) nM/ml in 96 well Fluorescence plate at Excitation and Emission wavelengths as 485 nm and 538 nm respectively and the graph for the five increasing DOX concentrations against the respective fluorescence readings was obtained in a linear fashion. The standardization for DOX (DOX calibration curve) carried out was used to calculate the intracellular drug concentration in the 7 days treated cell lysate as well as first day treated and control cell lysate accordingly.

Curcumin Co-Treatment

The curcumin treatment was started in the same flask in which DOX treatment was started at the same time. The two flasks with (70)% confluency were selected for this purpose. One flask was treated with increasing concentration of DOX after every 24 hours as well as the same flask was treated with 1μM curcumin concentration daily till the end of the 7 days treatment. The other 7 days DOX treatment flask was also treated with increasing DOX concentration for 7 days and 10μM curcumin concentrations daily as co-treatment till the end of the DOX treatment. Along-with this whole treatment process, one more flask was treated with only increasing concentration of DOX continuously for 7 days. So, in total 3 flasks were treated in this case. Also, the control flask was taken as normal HT29 cells cultivated in the flask. This treatment was repeated several times to ensure contamination free treatment.

Results

1. HT29 cell culture: HT29 cells were sub-cultured after attaining the 80% confluency (Figure 1)

2. 7 Days Treatment: In case of 7 days DOX treatment, HT29 colon cancer cells were treated after every 24 hours with increasing concentrations of Doxorubicin Hydrochloride continuously for 7 days period, also called as short term treatment. The cancer cells were treated for consecutive 1 week in the range of 0.23μM/L-1.6μM/L of DOX. The images were regularly captured under the inverted microscope as well as the dead cell count was checked with the help of Trypan blue assay using haemocytometer after every 24 hours from the respective flasks of the drug treatment as part of the project, The images were taken to observe the cell density change in the flasks during 7 days drug treatment (Figure 2).

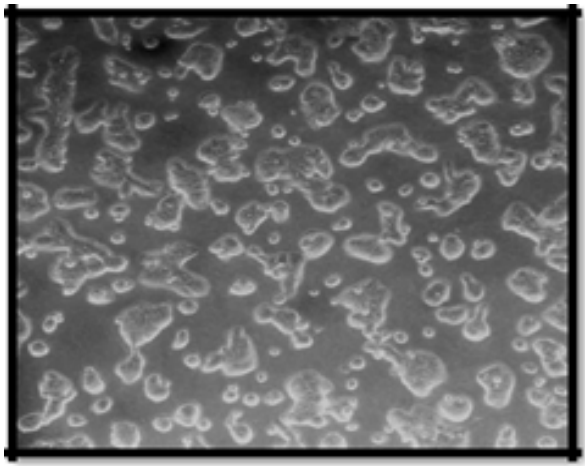

Figure 1: This figure shows the high i.e. 80% confluency of HT29 cells for passage purpose.

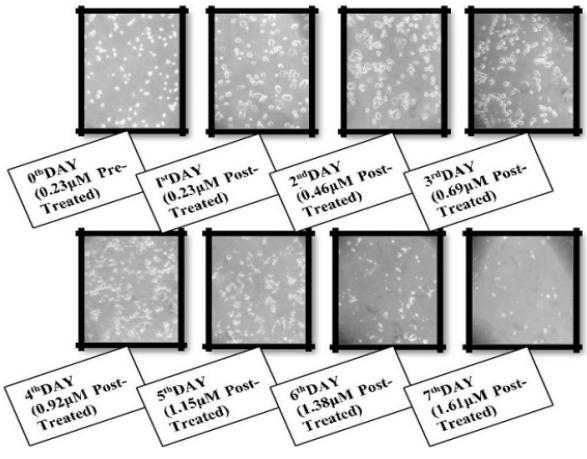

Figure 2: 7 days Dox post- treated cells images (24 hours post-treatment).

The 7 days treatment was started next day after passaging the normally cultured HT29 cell line. The cells were continuously treated after every 24 hours with the increasing concentrations of Doxorubicin Hydrochloride till the end of the 7th day. The following tabulation provides the total estimation of number of dead cells counted during the 7 days treatment (Table1)

The number of dead cells per ml multiplied by 10^4 which gives us the exact dead cell count after each day of treatment. The figure (Figure 3) shows drug concentration versus number of dead cells estimated.

3. **DOX calibration curve:** The following five different concentrations of Doxorubicin hydrochloride drug were used to obtain a standard graph (Table 2). The DOX concentrations were dissolved in lysis solution accordingly in order to make the final volume of 200µl for each concentration. The final volume of 200µl was transferred to 96-well fluorescence plate and the plate was subjected to fluorescence measurement. The excitation and emission wavelengths used are 485nm and 538nm respectively. The figure (Figure 4) shows concentration versus Fluorescence intensity.

4. **Doxorubicin Accumulation Assay:** Doxorubicin accumulation assay was carried out for 5 different cell lysate samples i.e. one sample as control (HT29 cells),two samples of first day post-treated cells (0.23µM DOX) and two samples of 7 days post-treated cells (1.61µM DOX).The intracellular DOX concentration was calculated using fluorimetric values obtained for each sample. The blank fluorescence value was subtracted from each value of the samples. The following data were calculated using different formulae to measure the unknown concentration. The following table shows the intracellular drug concentration at two different time periods of 7 days DOX treatment (Table 3)

S. No	Number of Days in 7 Days Treatment	Concentration of Dox Treatment (Mm)	Total Number of Dead Cells (Cells/Ml)
1	Day 1	0.23	12.5×10^4
2	Day 2	0.46	16.5×10^4
3	Day 3	0.69	33.5×10^4
4	Day 4	0.92	54×10^4
5	Day 5	1.15	79×10^4
6	Day 6	1.38	45×10^4
7	Day 7	1.61	29.5×10^4

Table 1: It shows the dead cell count after every 24 hours of treatment continuously for 1 week.

S. No	DOX concentrations used (Nmol/Ml)
1	0.2
2	1
3	3
4	5
5	7

Table 2: This table shows the 5 different concentrations used for DOX calibration curve.

S. No	Type of Sample	DOX Concentration (nmol/ml)	DOX Accumulation (nmol/mg protein)
1	Control (HT29 cells)	0.0	0.0
2	First day DOX post- treated (live cells (0.23 µM DOX))	2.85	1.83
3	First day DOX post- treated (dead cells (0.23 µM DOX))	0.1	0.24
4	7 days DOX post- treated (live cells (1.61 µM DOX))	1.45	3.207
5	7 days DOX post- treated (dead cells (1.61 µM DOX))	0.6	2.083

Table 3: This table shows the Intracellular concentration of DOX in the: 1) Control cell sample. 2) First day DOX post-treated live sample. 3) First day DOX post-treated dead cell sample. 4) 7 days DOX post-treated Live sample and 5) 7 days DOX post-treated Dead cell sample.

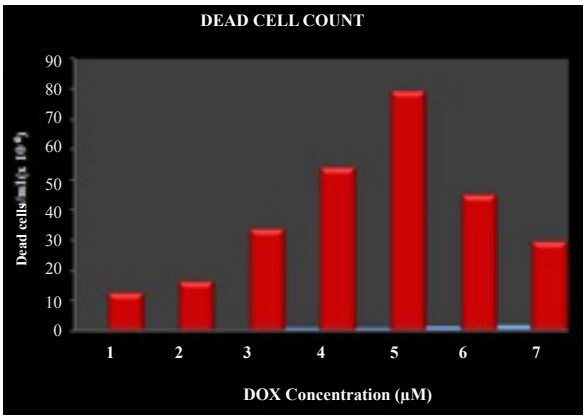

Figure 3: Dead cell count during 7 days DOX treatment.

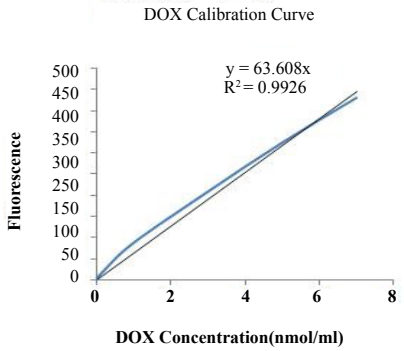

Figure 4: This graph shows the linear curve in case of DOX concentration *vs.* fluorescence.

Curcumin Co-Treatment

Curcumin co-treatment was performed on HT29 cells cultivated in the flask. In this case, the flasks for analysis were selected as 7 days DOX treated live and dead cells", "7 days DOX treated plus 1µM curcumin co-treated live and dead cells", "7 days DOX treated plus 10µM curcumin co-treated live and dead cells, "First day DOX treated live and dead cells", First day DOX treated plus 1µM curcumin co-treated, "First day DOX treated plus 10 µM curcumin co-treated live and dead cells" and control sample was taken as normally cultivated HT29 cells. The range of concentration for DOX treatment was used as 0.23µM-1.61µM. This process was performed using the fluorimetry method in which fluorescence values for DOX concentration in the lysate samples were recorded and further statistical analysis was done with the aid of the DOX calibration curve. In this method, comparison for sensitization was done between first day DOX post treated and 7 days DOX post treated live and deal HT29 cell samples and also curcumin co-treated live and dead HT29 colon cancer cell samples were compared in order to observe the sensitization of multidrug resistance

Treatment type	Sample name	DOX concentration (nmol/ml)	Protein estimation (mg/ml)	DOX accumulation (nmol/mg)
Control (Ht29 cells)	Control	0.00	4.16	0.00
DOX Treated	7 Days treated (Live)	2.60	0.63	4.11
	7 Days treated (Dead)	0.70	1.15	0.61
	1st Day treated (Live)	0.30	3.67	0.08
	1st Day treated (Dead)	0.10	0.70	0.14
DOX+1 μM Curcumin	7 Days treated (Live)	2.65	0.58	4.54
	7 Days treated (Dead)	2.00	1.54	1.30
	1st Day treated (Live)	1.30	1.59	0.81
	1st Day treated (Dead)	0.10	0.93	0.11
DOX+10 μM Curcumin	7 Days treated (Live)	4.15	0.66	6.33
	7 Days treated (Dead)	3.75	0.67	5.61
	1st Day treated (Live)	0.40	3.15	0.13
	1st Day treated (Dead)	0.10	0.88	0.11
7 Days Treatment=0.23 μM-1.61 μM DOX 1st Day Treatment=0.23 μM DOX				

Table 4: This table provides with the calculation and estimation of DOX accumulation based on the Intracellular concentration of DOX in the samples, 1) control cell sample (HT29 cells). 2) 7 days DOX post-treated live and dead cell samples. 3) 7 days DOX post-treated and 1 μM curcumin co-treated live and dead cell samples. 4) 7 days DOX post-treated and 10 μM curcumin co-treated live and dead cell samples. 5) 1st day DOX post-treated live and dead samples. 6) 1st day DOX post-treated and 1 μM curcumin co-treated live and dead cell samples. 7) 1st day DOX post-treated and 10 μM curcumin co-treated live and dead samples.

developed by DOX treatment (Table 4).

Discussion

The most common property of cancer cells being confirmed so far is to develop the ability to resist the cytotoxic activity of a vast range of drugs which in turn results in Multidrug resistance (MDR) [24-30]. These drugs may or may not be related to each other in their structure as well as function. There is enormous number of factors contributing to the development of multidrug resistance. Among them, the most important one is the over-expression of a plasma membrane ATPase called P-glycoprotein (P-gp) [31,32]. This enzyme helps in the outward movement of active drugs and decreases their concentration inside the cancer cells and as a result the cytotoxic efficacy of the respective drug gets reduced [33].

Human colon cancer cell line HT-29 was used to generate the Multi Drug resistant cells (MDR-HT29) by giving the continuous 24 hour exposure to the cells with increasing concentrations of DOX (0.23μM to 1.61μM) for 7 days. Doxorubicin being a highly cytotoxic drug, hence, in case of 7 days DOX treatment in HT29 colon cancer cells, there was a gradual increase observed in the dead cell count continuously up to 5th day and after the 5th day till the end of the treatment; the dead cell count got decreased [34]. In the DOX accumulation assay, the first day post treated cells showed high amount of drug inside the cells as compared to 7 days treated cells, which indicates that the activation of the ABC transporter protein takes place at some point near to the 7th day treatment and the drug is transported out of the cells in-turn expressing the resistance to the drug. In case of DOX as well as curcumin co-treatment, there were significant changes in the cell density as well as dead cell count was observed to get increased gradually during curcumin co-treatment thus showing the process of sensitization of multidrug resistant HT29 colon cancer cells due to treatment with curcumin [35]. Since Doxorubicin being a naturally fluorescent compound, with the help of this extremely supportive property of Doxorubicin drug, it was very helpful to determine the accumulation of Doxorubicin inside the DOX treated cells to confirm the acquired chemo resistance against this chemotherapeutic drug.

Conclusion

The 7 days DOX treatment in human colon cancer cell line HT29 was carried out and also curcumin co-treatment was started in the meantime. Due to less survival rate of HT29 cells at the end of 7 days treatment, the DOX treatment was repeated several times to ensure high accuracy in the experiment. The long term treatment was also repeated for obtaining resistant cells by treating the cells with 68nM/L DOX concentration after every 24 hours. DOX accumulation assay was performed which showed the moderate drug concentration inside the 7 days treated live cells as well as dead cells. The DOX accumulation assay results for 7 days treated cells were compared with the first day DOX post treated as well as control HT29 cells to confirm the drug accumulation in all the samples. This analysis confirmed the activation of drug efflux pump P-gp at the end of the drug treatment by showing less intracellular drug concentration in 7 days post-treated cells as compared to first day post-treated cells. Further, the curcumin co-treatment was carried out repeatedly in order to ensure high accuracy as well as contamination free process, to estimate the DOX accumulation inside the curcumin co-treated HT29 colon cancer cells and the effect of curcumin on the resistance during the co-treatment. Curcumin co-treatment showed the gradual increase in the amount of dead cells collected after every 24 hours DOX post-treatment and curcumin co-treatment, which clearly showed the sensitization of chemo-resistance developed due to the continuous DOX treatment by activating the efflux pump, P-gp. Thus, this experiment came up with some important concepts to understand in case of using DOX as well as curcumin (phenolic compound) together in the treatment of colon cancer. Further processes such as western blot analysis, etc. could be very much helpful to investigate the effect of Curcumin on COX2 expression as well as NfkB expression which will be very much supportive for researchers to develop highly specific and accurate therapeutic treatment methods in order to control the colorectal cancer at global level.

Conflicts of Interest

The authors declare no conflicts of interest.

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