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In vitro Cytotoxicity, Apoptosis, Effects on Cell Cycle Kinetics and Schedule-Dependent Effects Induced by Paclitaxel on C6 and CHO-K1 Cell Lines

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ABSTRACT

Exposure of C6 and CHO-K1 cells to different concentrations of the antineoplastic drug paclitaxel resulted in a loss of cellular viability. The percentage of surviving cells fell significantly after 48 hours of treatment and IC_{50} values observed were between 0.5 to 0.75 and 0.25 to 0.75 µg/ml in C6 and CHO-K1 cells, respectively. No significant cytotoxicity was observed after 24 hours of treatment and cells incubated at higher concentrations of paclitaxel showed increased survivability. Paclitaxel induced apoptosis by caspase 3/7 activation and caused accumulation of cells in the G2/M phase of the cell cycle. Upon fluorescent microscopy, both the cell lines lost the morphology, confluence and adherence at 24 hours but effects were much more pronounced at 48 hours of treatment. The *in vitro* data suggested that paclitaxel is highly effective when there is prolonged exposure of tumor to the drug rather than increasing the intratumoral or biophasic concentration of the drug.

Key words: Apoptosis, CHO-K1, Cytotoxicity, C6, G2/M phase, Paclitaxel.

INTRODUCTION

Paclitaxel is a miraculous drug, one of most extensively studied, complex semi-synthetic diterpine alkaloid, an antineoplastic agent obtained primarily from the European Yew (*Taxus Baccata*) and the Pacific Yew (*Taxus Brevifolia*), widely used against lung, advanced breast and ovarian carcinomas (Bhat *et al.* 2016). It is also being employed for the treatment of squamous cell carcinomas of head and neck, prostrate, endometrial, gastroesophageal cancers and recent studies have shown that paclitaxel can also be used for the treatment of leukemia, lymphoma and sarcoma Khanna *et al.* (2015).

Paclitaxel is a microtubule-stabilizing anticancer agent, inducing mitotic arrest by suppression of microtubule dynamics (Schiff *et al.* 1979). The over stabilization of microtubules results in the inhibition of normal re-organization of the network, leading to the cytotoxic effects in a subset of the arrested population (Diaz *et al.* 1993). However, recent studies have shown that paclitaxel does not acquire sufficient intra-tumoral concentrations to cause mitotic arrest and it has been observed that the cytotoxic effects may be due to chromosome misaggregation on multipolar spindles (Beth, 2014).

Taxanes have revolutionized the cancer chemotherapy and have shown great results when used in combination with other cytotoxic agents but the cytotoxic potential of paclitaxel against gliomas has not been elucidated conclusively (Bhat *et al.* 2016). Gliomas account for majority of primary malignant brain tumors and are associated with very poor survival rates. It is of utmost importance to formulate formulations achieving profound cytotoxic concentrations inside glioma tumoral cells, with minimal damage to normal cells (Bhat *et al.* 2016).

The present study was undertaken to study *in vitro* cytotoxic effects, apoptosis, effects on cell cycle kinetics and schedule dependent effects induced by paclitaxel on C6 (*Rattus norvegicus* glioma cell line) and CHO-K1 cell lines (Chinese Hamster ovarian carcinoma).

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MATERIALS AND METHODS

Paclitaxel

Paclitaxel (10 mg, Catalogue No. RM9750) was obtained from HIMEDIA and stored at 2-8°C.

Cell lines and Cell culture

C6 (glioma cells) cells were obtained from National Centre for Cell Science, Pune-India and cultured in DMEM (Sigma Aldrich) supplemented with 10% heat inactivated fetal bovine serum and 1% antibiotic-antimycotic (Sigma Aldrich). CHO-

K1 (Chinese hamster ovary cells) cells were obtained from CSIR-Indian Institute of Integrative Medicine, Jammu-India and were cultured in HAM'S-F12 medium (Sigma Aldrich), supplemented with 10% heat inactivated fetal bovine serum (Sigma Aldrich) and 1% antibiotic-antimycotic (Sigma Aldrich). Both the cell lines were maintained at 37°C in 5% CO_2 humidified atmosphere (Katoch *et al.* 2012).

Sulforhodamine B assay

The level of cytotoxicity was measured using the Sulforhodamine B (SRB) method (Walia *et al.* 2012). The viable cells were seeded in the growth medium (100 µL) into 96 well microtiter plates ($5X10^4$ cells per well) and allowed to adhere overnight. The test sample, paclitaxel, was dissolved in DMSO and several dilutions (0.25, 0.50, 0.75, 1.25, 2.50 and 5 µg/ml) of the test compound in 100 µl of respective complete media was added to the wells. The cell culture alone supplemented with complete medium was used as a negative control, while as vinblastine (1µM) was used as a positive control for the assay. After 24 and 48 hours, 50 µl of 50% trichloroacetic acid was added to the wells and the plates were incubated at 4°C for one hour. The TCA treated cells were washed extensively with water and air dried. Subsequently, 100 µL of the SRB solution (in 1% Acetic acid) was added to each well at room temperature. After standing for 30 min, the wells were washed 5-6 times with 1% Acetic Acid and air dried. The bound dye was dissolved in 10 mM tris base (100 µl/well). The absorbance was measured using microplate reader (BioTeK Synergy H1 Hybrid Reader) at the wavelength of 540 nm (Joshi *et al.* 2012). The growth inhibition rate was calculated using the following formula:

$$\% \text{ Cell inhibition} = 100 - \{(A_t-A_b)/(A_c-A_b)\} \times 100$$

Where, A_t, A_b and A_c represent absorbance value of test compound, blank and control, respectively.

Apoptotic (Caspase 3/7 activity) assay

The cells were plated in 96-well plates at 2×10^4 density. After 3 hours of treatment of cells, caspase activity was detected against C6 and CHO-K1 cell lines using different concentrations of paclitaxel (0.50, 0.75, 1.25 µg/ml against CHO-K1and 1.0, 1.5 and 2.5 µg/ml against C6). The total caspase activity was detected using the Caspase-Glo® 3/7 Assay kit (Promega, Catalogue No. G8090). The medium alone was used as a negative control for the activity, whereas the cell cultures exposed only to DMSO (1%) was used as a positive control. The activity was measured according to the supplier's instructions provided with the kit. Caspase-3/7 activity was measured in net relative luminescence units (RLU) of respective incubated cells in the 96-well plates and was measured using microplate reader (BioTeK Synergy H1 Hybrid Reader) as per the recommended protocol. Following formula was used to calculate caspase 3/7activity in relative luminescence units (RLU):

RLU = Luminescence (samples) – Luminescence (blank)

Cell cycle analysis

Cell cycle analysis was done by Muse™ Cell Cycle Kit, according to the manufacturer's instructions (Catalogue No. MCH100106). Ethanol-fixed cells were treated with a premixed Muse™ Cell Cycle Reagent and incubated at room temperature for 30 minutes. The concentrations of paclitaxel applied in C6 and CHO-K1 cells were 0.75 and 0.5 µg, respectively.

Morphological changes

The morphological changes of C6 and CHO-K1 cells treated with paclitaxel for 24 and 48 hours were observed by using inverted fluorescent microscope (Nikon Eclipse T_i) at 10X magnification.

RESULTS AND DISCUSSION

Sulforhodamine B assay

Paclitaxel showed highest inhibition on C6 cells at concentration of 1.25 µg/ml and on CHO-K1 cells at 0.5 µg/ml after 48 hours of treatment. No significant changes were obtained after 24 hours in both the cell lines. The IC_{50} values of Paclitaxel were observed between 0.5 to 0.75 µg/ml in case of C6 cells and 0.25 to 0.75 µg/ml in CHO-K1 cells. The results are depicted in Table 1 and 2.

In both cell lines the percentage of cytotoxicity increased with increase in the time dependent exposure of cells to paclitaxel. This schedule dependent effect of paclitaxel may be because longer durations of drug exposure may allow greater proportion of cells to cycle into the susceptible phase (Georgiadis *et al.* 1997). The results showed that cell death occurs in cells with slower kinetics rather than using increasing concentrations of paclitaxel suggesting that cells must spend sufficient time in presence of paclitaxel, as longer durations of drug exposure may induce lethality in the greater proportion of cells (Katherine *et al.* 2013) and previous studies have shown that prolonged exposure of clinically relevant concentrations of paclitaxel may lead to the formation of chromosome misaggregation on multipolar spindles, causing lethality (Lauren *et al.* 2014). The results of this assay are in agreement with previous studies that analyzed the growth inhibiting effects of paclitaxel and found concentration of paclitaxel needed to inhibit tumour cell growth by 50% (IC_{50}) was typically in the nanomolar/L range (Maria, 2014).

Apoptotic (Caspase-3/7 activity) assay

Caspases are a family intracellular proteases (cysteinyl-dirested aspartate specific proteases) (Riedl and Shi, 2004) that regulate the process of programmed cell death in response to proapoptotic signals. According to their mechanism of action, caspases can be classified as initiator (caspase -8 and -9) and executioner caspases (caspase -3.-6 and -7). Initiator caspases activate executioner caspases that eventually leads to apoptosis. Activation of caspases -3 and -7 is regarded as a hallmark of apoptosis (Mcllwain *et al.* 2013 and Portera and Janike, 1999).

Table 1: Cytotoxic effect of paclitaxel on C6 cell line after 24 and 48 hours of treatment.

Well identity	Blank 540	Mean ± S.D	% Cytotoxicity	
			(24 hours)	(48 hours)
Cells and media	1.283	1.332±0.057		
	1.394			
	1.318			
Positive control	0.498	0.446±0.054	66.5	S.P
	0.391			
	0.449			
0.25	1.152	1.124±0.025	15.6	17.3
	1.104			
	1.116			
0.50*	1.093	1.078±0.029	19.06	43.3
	1.095			
	1.044			
0.75*	1.121	1.134±0.101	14.8	56.5
	1.241			
	1.040			
1.25*	0.567	0.478±0.078	9.4	67.3
	0.424			
	0.442			
2.5	0.516	0.477±0.036	9.6	31.3
	0.470			
	0.446			
5.0*	0.474	0.485±0.034	8.1	43.9
	0.523			
	0.458			

S.P Represents for Slight Proliferation; *Represents effective concentrations close to IC_{50}.

Table 2: Cytotoxic effect of paclitaxel on CHO-K1 cell line after 24 and 48 hours of treatment.

Well identity	Blank 540	Mean ± S.D	% Cytotoxicity	
			(24 hours)	(48 hours)
Cells and media	0.753	0.780±0.024		
	0.798			
	0.791			
Positive control	0.162	0.235±0.086	69.8	88.8
	0.213			
	0.330			
0.25*	0.628	0.721±0.060	7.5	43.9
	0.777			
	0.729			
0.5*	0.679	0.754±0.072	3.3	65.5
	0.823			
	0.762			
0.75*	0.732	0.687±0.080	11.9	60.8
	0.734			
	0.594			
1.25	1.076	1.054±0.066	7.7	25.7
	0.980			
	1.107			
2.5	1.022	0.887±0.122	22.3	13.6
	0.783			
	0.857			
5.0	1.097	1.021±0.072	10.6	4.1
	0.954			
	1.011			

*Represents effective concentrations close to IC_{50}.

Paclitaxel showed highest relative luminescence unit at the concentration of 2.5 µg/ml against C6 and at 1.25 µg/ml against CHO-K1 cell lines. The results depicted in Table 3 and 4, show significant activation of caspases and are in agreement with Byrne *et al.* (2006), who found that paclitaxel elicited a caspase response at the IC_{50} value of 20-30 µM in normal human mammalian epithelial cells (HMECs) and mammary tumor cell line (MDA-MB-231). In this present study, caspases-3/7 were dramatically activated at 48-72 hours with increasing paclitaxel concentration and enhanced activity of caspases-3/7 was evidently verified by the measurement of the cleavage of poly(ADP-ribose) polymerase (PARP). The significant increase in the activity of caspases-3/7 suggested the schedule dependent induction of apoptosis (Jelinek *et al.* 2015). However, previous studies have shown that caspase activation may not be the only mechanism of apoptosis induced by paclitaxel and may also be dependent upon the intracellular concentrations of the drug, for instance, apoptosis may occur by p53 up-regulation instead of following the caspase pathway Kim *et al.* (2013), especially at low concentrations at which caspase reactions are not significantly active in inducing apoptosis (Tan *et al.* 2002).

Cell cycle analysis

Muse™ Cell Cycle Assay utilizes the differential staining of cells based on the DNA content. The nuclear DNA stain, prodidium iodide, intercalates with the DNA causing fluorescence. Cells in G_o/G_1 state contain diploid set of chromosomes and as the cells begin cycling the fluorescence increases and doubles in the G_2/M phase, followed by the cellular division (Ho *et al.* 2004).

Paclitaxel at concentration of 0.75 µg/ml caused an accumulation of the cells in G_2/M phase by about 41% and that of control by about 20.1%, in C6 cells. 0.5 µg/ml concentration was applied to CHO-K1 cells and it caused an accumulation of cells in G_2/M phase by about 57.7% and that of control by about 25.6%. These results suggest that G_2/M arrest and a decrease in the population of cells in G_1 phase, was induced by paclitaxel in both the cell lines. These results are in agreement with previous studies, for instance, Juliet *et al.* (2014) showed 25 nM and 50 nM of paclitaxel induced G_2M blocked in MDA-MB-231 and ZR75-1 cells.

The concentrations used in the given experimental were sufficient to induce caspase activation and interact significantly with the spindle-proteins leading to mitotic arrest. However, previous studies also reveal that paclitaxel can induce G_1 arrest at low intracytoplasmic concentrations and may be because of Bcl-2 phosphorylation (Kim *et al.* 2013) and chromosome misaggregation on multipolar spindles (Lauren *et al.* 2014).

The results are depicted in Table 5 to 8 and Fig 1 and 2.

Morphological changes

The morphological changes in C6 and CHO-K1 cell lines treated with 0.75 and 0.5 µg/ml of paclitaxel, respectively, for 24 and 48 hours, were observed, as determination of key adherent cell culture characteristics such as confluency morphology and cell density are necessary for the evaluation of experimental outcomes.

Paclitaxel showed increased potential of cytotoxicity in a time dependent manner on both the cell lines. Untreated C6 cell line represented normal neuronal morphology, confluency and cell density (plate 1) however on treatment

Table 3: Caspase activity of paclitaxel against C6 cancer cell line.

Well identity	Conc.(µg/ml)	Mean	Relative luminescence units
Blank		5064.33	
DMSO	1(µl)	109028.30	103963.97
Paclitaxel	1	139073	134008.67
	1.5	158561.30	153496.97
	2.5	174590.33	169526

Table 4: Caspase activity of paclitaxel against CHO-K1 cancer cell line.

Well identity	Conc.(µg/ml)	Mean	Relative luminescence units
Blank		5067.67	
DMSO	1(µl)	24754.33	19677.65
Paclitaxel	0.50	34701.67	29624.99
	0.75	29334	24257.32
	1.25	38453.67	29776.99

Table 5: Cell cycle kinetics of untreated C6 cell line.

	G_0/G_1	S	G_2/M	Debris
% Gated	66.3	7.9	20.1	67.3
Mean	2160.8	3937.2	4782.5	423.1
% CV	15.4	3.8	9.2	298.8

for 24 hours cell line lost its morphology and decreased confluency and cell density was observed (plate 2). The effects were much more pronounced at 48 hours (plate 3) of treatment.

CHO-K1 is an epithelial type of cell line (plate 4), so its cell size is smaller than C6, as evident from the respective untreated plates. The treatment for 24 hours caused a decrease in cell line density but retained some ability of adherence with slight reduction in confluency (plate 5). It may be because of the fact that concentration of paclitaxel used was less than that of used in C6. CHO-K1 showed much more pronounced decrease in adherence, confluency and density at 48 hours (plate 6).

Adherent property is an important feature of C6 and CHO-K1 and upon treatment both the cell lines lost their adherence thereby decreasing the cell density and confluency indicating cytotoxic effects on C6 and CHO-K1 (Jaccard *et al.* 2013).

Table 6: Cell cycle kinetics of treated C6 cell line.

	G_0/G_1	S	G_2/M	Debris
% Gated	48.9	5.1	41.0	87.3
Mean	2853.9	3941.3	5120.2	326.2
% CV	12.6	3.8	8.2	197.9

Table 7: Cell cycle kinetics of untreated CHO-K1 cell line.

	G_0/G_1	S	G_2/M	Debris
% Gated	64.8	4.7	25.6	68.6
Mean	2530.1	3407.0	4414.8	113.7
% CV	9.4	3.5	10.2	475.0

Table 8: Cell cycle kinetics of treated CHO-K1 cell line.

	G_0/G_1	S	G_2/M	Debris
% Gated	28.2	3.3	57.7	81.1
Mean	2545.9	3424.1	4586.6	135.0
% CV	10.3	3.6	9.6	544.3

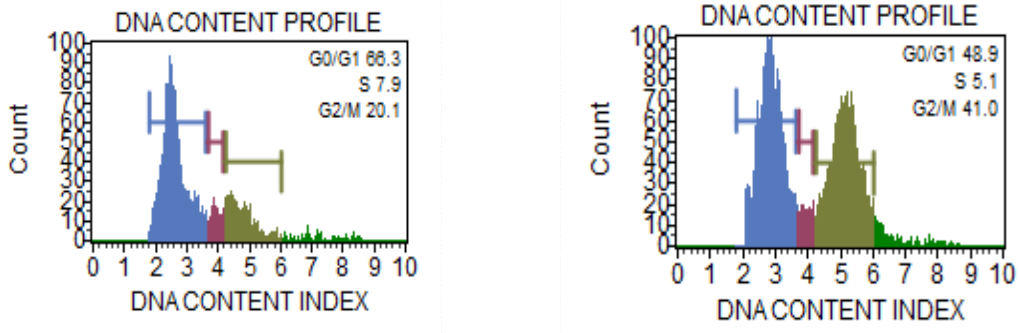

Fig 1: DNA content index of treated and untreated C6 cell line.

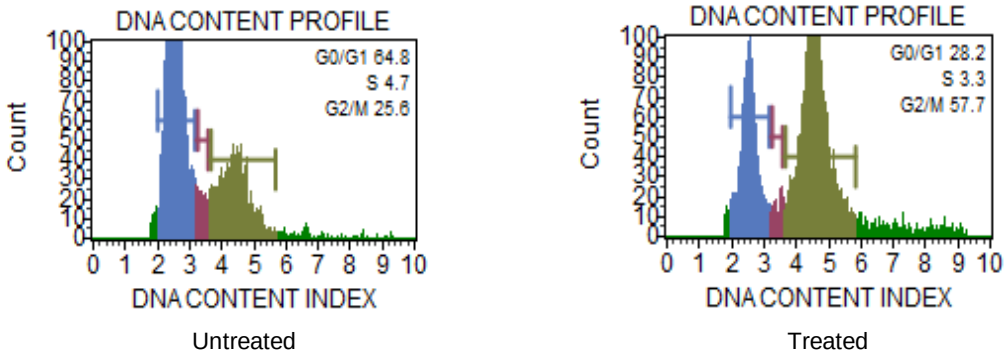

Fig 2: DNA content index of treated and untreated CHO-K1 cell line.

FLOURESCENT MICROSCOPY OF C6 & CHO-K1 CELL LINES

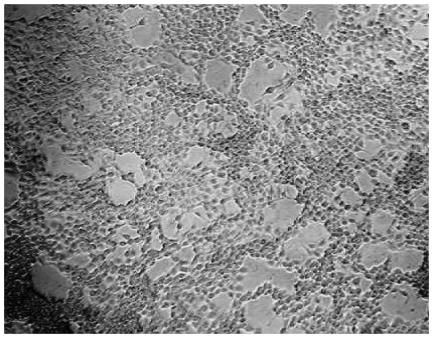

Plate 1: UNTREATED C6.

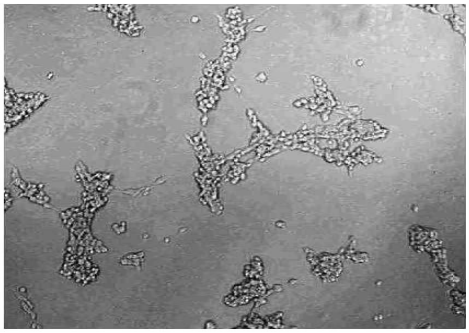

Plate 2: C6_24 hr.

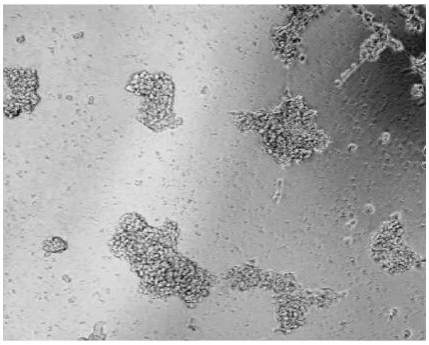

Plate 3: C6_48 hr.

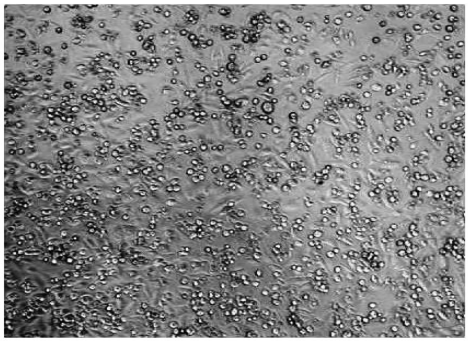

Plate 4: UNTREATED CHO-K1.

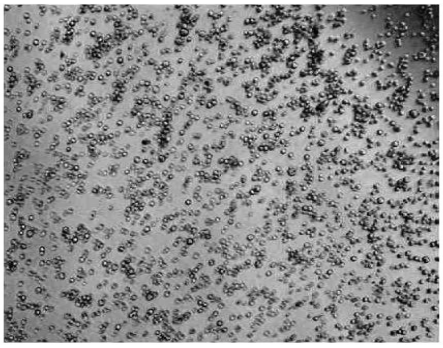

Plate 5: CHO-K1_24 hr.

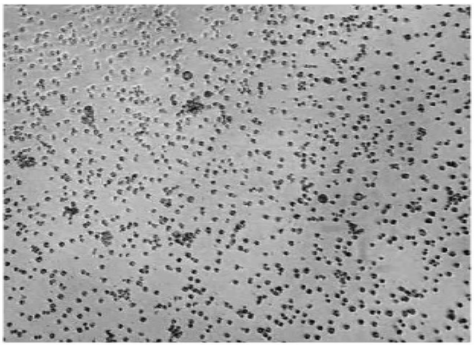

Plate 6: CHO-K1_48 hr.

CONCLUSION

Paclitaxel showed highest inhibition on C6 cells at concentration of 1.25 µg/ml and on CHO-K1 cells at 0.5 µg/ml after 48 hours of treatment. No significant changes were obtained after 24 hours in both the cell lines. Cells incubated in high concentrations of paclitaxel had increased survivability compared with cells treated with lower concentrations of the drug. The IC_{50} values of Paclitaxel were observed between 0.5 to 0.75 µg/ml in case of C6 cells and 0.25 to 0.75 µg/ml in CHO-K1 cells. Apoptosis was screened by employing Caspase-Glo® 3/7 Assay kit. Paclitaxel showed highest RLU at concentration of 2.5 µg/ml against C6 and at 1.25 µg/ml against CHO-K1 cell line, clearly indicating paclitaxel leads apoptosis by caspase 3/7 activation. Cell cycle analysis was done by Muse™ Cell Cycle Kit and G_2/M phase arrest was observed in both the cell lines. Upon fluorescent microscopy, both the cell lines lost their morphology, confluency and adherence after 24 hours but the effects were much more pronounced after 48 hours of treatment.

The *in vitro* data suggests that paclitaxel is highly effective when there is prolonged exposure of tumour to the drug rather than increasing the intrumoral or biophasic concentration of drug.

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Conflict of Interest

The authors do not present any conflict of interest.

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