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Cytotoxicity of neoplastic drugs Gefitinib, Cisplatin, 5-FU, Gemcitabine, and Vinorelbine on human cervical cancer cells (HeLa)

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Abstract
A recent study reports that more than 99% of cases of cervical cancer worldwide contain HPV DNA. Hence, treatment options for cervical cancers are difficult due to multiplicity of the disease. Chemotherapy uses strong anti-cancer chemicals to kill cancer cells but to kill the viruses clinicians administer combination of drugs or higher doses of chemotherapy to control advanced cervical cancer and this practice causes severe side effects. Numerous cancer patients fail standard chemotherapy or develop resistance to chemotherapy during the course of treatment. Hence the aim of this investigation was to determine the chemo sensitivity of the five commonly used neoplastic drugs such as Geftinib, Cisplatin, 5-FU, Gemcitabine and Vinorelbine *in vitro*, and compare its toxicity on cervical cancer cells (HeLa) using lymphocytes (nucleated cells) as controls. Cytotoxicity *in vitro* was determined using the MTT assay; LC50 for all the drugs was calculated by regression equation. The morphological change of cells was recorded using Inverted Microscopy. DNA damage studies by comet assay determined the extent of single strand breaks in the DNA and these results were statistically determined using Standard deviation and compared with various treatments in cancer cells (HeLa) and control cells. All the results were statistically analyzed and recorded. From these studies we could determine that cisplatin was the most toxic drug and vinorelbine was least toxic. The order of toxicity (LC50) of the neoplastic drugs was Cisplatin (13µM) > Gefitinib (20µM) > Gemcitabine (35µM) > 5-FU (40µM) > Vinorelbine (48µM). Further, we could determine the toxicity of the combination of drugs using sub-lethal doses of each drug.

Keywords: HeLa cells; *In vitro* toxicity; DNA damage; Geftinib; Cisplatin; 5-FU; Gemcitabine; Vinorelbine; MTT-Assay; Comet Assay.

Introduction

Among all cancers cervical cancer is the second most common malignant neoplasm affecting women worldwide, with approximately 500,000 new cases diagnosed and 280,000 deaths each year according to WHO report. The highest incidences occur in the developing world, where the incidence of HPV is often prevalent. The virus infects the cells of the cervix and causes precancerous cellular changes (dysplasia) that can progress. Larger, deeper lesions (severe dysplasia) are more likely to progress to cervical cancer. Treatment options for cervical cancer depend on several factors, such as the stage of the cancer and other health related problems (Jemal *et al.*, 2006). In several developing countries, Pap test and routine screening for this preventable cancer is unavailable. As a result, cervical cancer remains the leading cause of cancer deaths among women in developing countries.

Chemotherapy is the standard therapy for advanced or metastatic disease and anti-tumor activity of drugs depends on the mechanism of action of how these agents are effective in killing the tumor cells or in preventing the growth of tumor cells. Single agent plays critical role in the local and regional control of malignant tumors. However, its efficacy can be limited by a number of factors including increased toxicity, normal tissue injury, drug resistance and increased side effects (Candelaria *et al.*, 2006; Umaznor *et al.*, 2006; Elst *et al.*, 2007; Gonzalez-Cortijo *et al.*, 2008). 5-Fluorouracil (5-FU) is currently being used as an anticancer drug to achieve optimal response and prolong the postoperative survival in patients of cervical cancer (Morris *et al.*, 1999; Thomas *et al.*, 2001). Studies have proven that Gemcitabine (2', 2'-difluorodeoxycytidine) is an anti-neoplastic agent that inhibits DNA synthesis, resulting in apoptosis. In addition to its established uses in pancreatic and non-small-cell lung cancer, the drug has been shown in clinical trials to be active against a wide variety of solid tumors (Carmichael *et al.*, 1998). Among the existing chemotherapeutic drugs cisplatin is considered to be the most active chemotherapeutic agent as a single agent or in combination (Vermorken *et al.*, 2001; Omura *et*

al., 1997). The relatively new drug Gefitinib (Iressa) is an orally active epidermal growth factor receptor tyrosine kinase inhibitor that blocks signaling pathways implicated in solid tumor growth and metastasis (Ranson *et al.*, 2004). Vinorelbine is of a new generation of *vinca* alkaloids, which exerts its biological effects by inhibiting microtubule assembly (Leveque *et al.*, 1996). The aim of the present investigation was to evaluate the cyototoxic and genotoxic effect of commonly used neoplastic drugs like Gefitinib, Cisplatin, 5-FU, Gemcitabine, and Vinorelbine on human cervical cancer cells (HeLa) in single modalities or in combinations and to compare the toxicity and DNA damage caused by these drugs. It is a common practice in medical oncology to use a combination of Cisplatin plus 5-FU for the treatment of cervical cancers. Hence, keeping these two drugs we have added one more new drug to see its effectiveness in cancer treatments. Such combinations have also been reported earlier by Fanning *et al.* (1995), Zanetta *et al.* (1999) and Budman *et al.* (2002). A comparative study would enable medical oncologists to decide the dosage levels of these drugs for their effective implementation in clinical practices. A follow up of the morphological features would confirm the levels of cell death, hence the need for this study.

Materials and Methods

Cell cultures

Human cervical carcinoma cells (HeLa) were obtained as a kind gift from Manipal University (India). HeLa were maintained in Dulbecco's modified Eagle's medium (D-MEM) plus 10% fetal bovine serum and penicillin (50 units ml^{-1})/streptomycin (50 µg ml^{-1}) (growth medium). All the chemicals were obtained from Sigma Chemicals (USA). Cultures were incubated in a humidified atmosphere of 5% CO_2 at 37°C. Rapidly proliferating cells were utilized for establishing cultures of experimental cells, which were allowed to incubate overnight prior to manipulation. Lymphocytes isolated from healthy non-smoking donors, were used as control (normal) cells.

Preparation of drug solutions for in vitro assays

Aqueous solutions of all the drugs were prepared in distilled water.

5-Fluorouracil (5-FU): was obtained from Dabur Pharma Ltd, India in aqueous form (1mg/1ml).

Gemcitabine: was obtained in powder form (40 mg/ml) (Intas Biopharmaceuticals, India).

Cisplatin: was obtained from Cadila Pharmaceuticals Ltd, India (Platin-50) in aqueous form (50mg in 50ml).

Vinorelbine: was obtained from Dabur Pharma Ltd, India (Vinelbine) in aqueous form (1mg/1ml).

Gefitinib: was obtained from Genex Pharma, Mumbai, India (Geftinat) as 250mg tablet.

Purification of lymphocytes

Short term lymphocyte cultures: About 3-5 ml fresh blood from volunteers with informed consent in heparinized collection vials was used for the experiments. Lymphocytes were isolated using HiSep Media (HiMedia, India) (as per the manufacturer's instructions). Briefly, HiSep media was added to the blood samples in the ratio 1:3 (media: blood) and centrifuged at 160 g for 20 min. The lymphocytes were then separated into fresh tubes and equal volume of PBS was added. This was again centrifuged at 140 g for 15 min for removal of HiSep Media. A second wash in PBS was given followed by centrifugation at 140 g for 15 min. The pellet was re-suspended in RPMI media, counted and plated in triplicates in 96-well microplates.

MTT assay

The MTT [3, (4, 5-Demethyl thiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay was performed as per standard protocol. In brief, HeLa cells were harvested and counted for viability using hemocytometer (Marienfeld, Germany). HeLa cells and lymphocytes were cultured in 24 well plates at a density of $5x10^6$ cells per well. The cells were treated with varying concentrations of Gefitinib, Cisplatin, 5-FU, Gemcitabine, and Vinorelbine. After 24 hrs, the cells were washed and treated with MTT. The formazan crystals were dissolved in 100µl of DMSO (Sigma, Aldrich). Plates were incubated in dark for 4 h, and the absorbance was measured at 570 nm using a micro titer plate reader (Bio Tek, USA). The experiment was repeated at least three times (Cartwright *et al.*, 1997; Dash *et al.*, 2003).

Growth inhibition is expressed as:

To determine the cell viability, percent viability was calculated [(absorbance of drug-treated) sample) / (control absorbance)] x 100. All the experiments were repeated at least three times.

DNA damage studies by Comet Assay

The DNA damage was determined using comet assay as described by (Singh *et al.*, 1988), with slight modifications (Jamil *et al.*, 2004). The concentrations used for comet assay were sub-lethal or low concentrations of the drugs, for all the various drugs. Slides prepared were in duplicates, the average values of comet tail lengths were analyzed using statistical methods.

Studies on morphological variations of cell lines using inverted microscope

Morphological changes in HeLa cell elicited by 5-FU, Cisplatin, Gemcitabine, Vinorelbine and Gefitinib were documented using inverted microscope (Labomed-USA) (Sunilson *et al.*, 2009).

Statistical analysis

The values of the comet assay in this study were expressed as means ±SEM from three experiments, i.e. data from three experiments were pooled and the statistical parameters were calculated.

Results

a) Studies on the toxicity of individual drugs

HeLa cells and lymphocytes showed growth inhibition in a dose dependent manner when treated with Cisplatin at concentrations ranging from 1-50µM. The LC-50 value of Cisplatin was found to be 13µM for HeLa and 20µM for lymphocytes (Figure-1). Morphological features observed using inverted microscope showed characteristic rounding of dying cells when treated with 13µM Cisplatin for 24hrs when compared with untreated control (Picture-F).

When both HeLa cells and lymphocytes were treated with 5-Fluorouracil, they showed variations in their growth inhibition curves (Figure-2), where we found that LC50 for HeLa cells was 20µM and 35µM for lymphocytes respectively. These results indicate that these cells responded in a dose dependent manner indicating that 5-Fluorouracil was moderately toxic to cancer cells than normal lymphocytes. The morphological changes observed using the inverted microscope (Picture-B) showed characteristic rounding off of dying cells on treatment with 20µM 5-Fluorouracil for 24hrs compared to untreated control.

Figure-3 illustrates growth inhibition curve of HeLa and lymphocytes after treatment with Vinorelbine in a dose dependent manner at concentrations ranging from 1-100µM. The LC-50 value of Vinorelbine was found to be 78µM for lymphocytes and 48µM for HeLa. Morphological features observed using inverted microscope (Picture-D) showed characteristic rounding of dying cells when treated with 48µM Cisplatin for 24hrs when compared with untreated control.

The cell viability effect of Gefitinib on lymphocytes and HeLa cells after treatment was found to be at 30µM and 20µM after 24hrs incubation (Figure-4). Morphological features observed using inverted microscope (Picture-E) showed characteristic rounding of dying cells when treated with 20µM Cisplatin for 24hrs when compared with untreated control.

Gemcitabine also showed its effect on lymphocytes and HeLa cells in a dose dependent manner. The LC-50 was found to be 50µM for lymphocytes and 35µM for HeLa after 24hrs incubation (Figure-5). Morphological features observed using inverted microscope (Picture-C) showed characteristic rounding of dying cells when treated with 35µM Gemcitabine for 24hrs when compared with untreated control. The order of toxicity of the neoplastic drugs was found to be Cisplatin > Gefitinib > Gemcitabine > 5-FU > Vinorelbine.

b) Studies on toxicity of combination of drugs

In these experiments we used the three drugs 5-FU, Cisplatin and Gefitinib. The results of the combination of drugs tested on HeLa cells are presented in Figure-6. These drugs were combined at their sub-lethal doses as determined by the MTT assay mentioned above. Mostly these doses represent less than LC_{20} values. The doses were combined in their increasing concentrations as shown in (Figure-6). We found the best combination was at “D” concentration of the combined drugs (Figure-6).

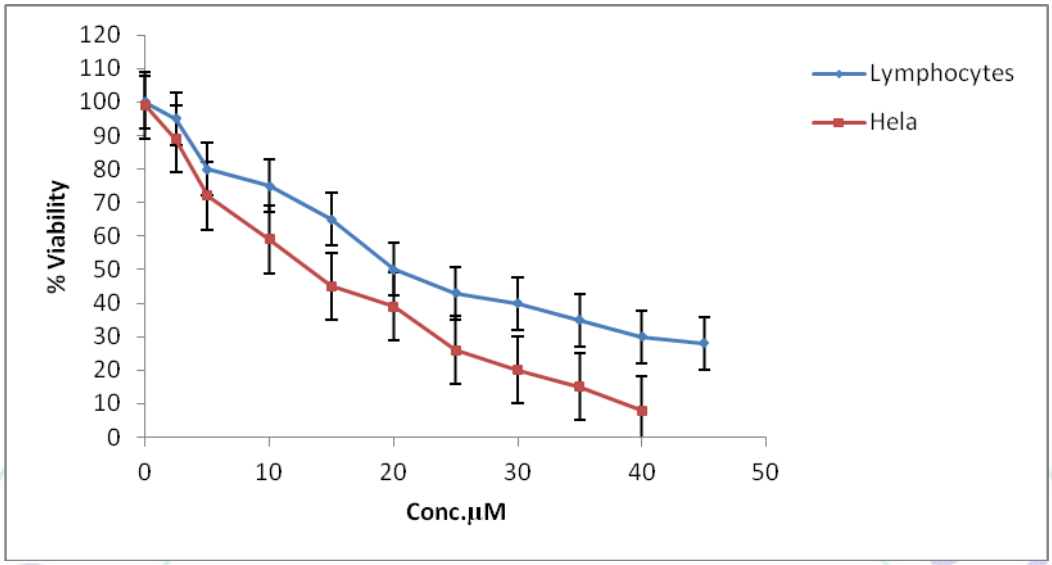


Figure 1: Dose dependent curve of lymphocytes (controls) and HeLa cells after treatment with Cisplatin at 24hrs. The LC-50 was 20µM for controls and 13µM for HeLa cells.

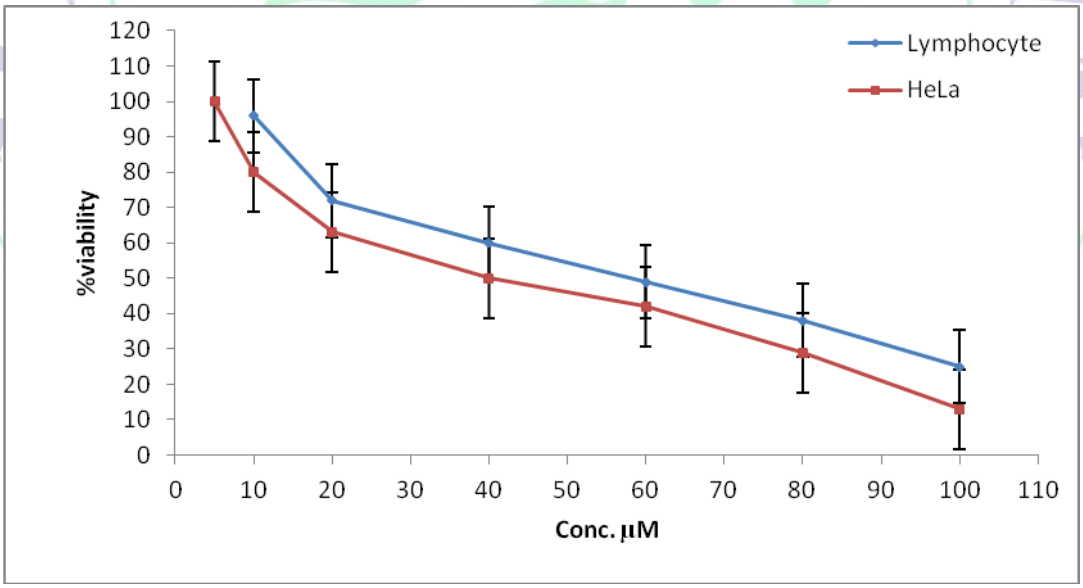


Figure 2: Dose dependent curve of lymphocytes (controls) and HeLa cells after treatment with 5-Fluorouracil at 24hrs. The LC-50 was 59µM for controls and 40µM for HeLa cells.

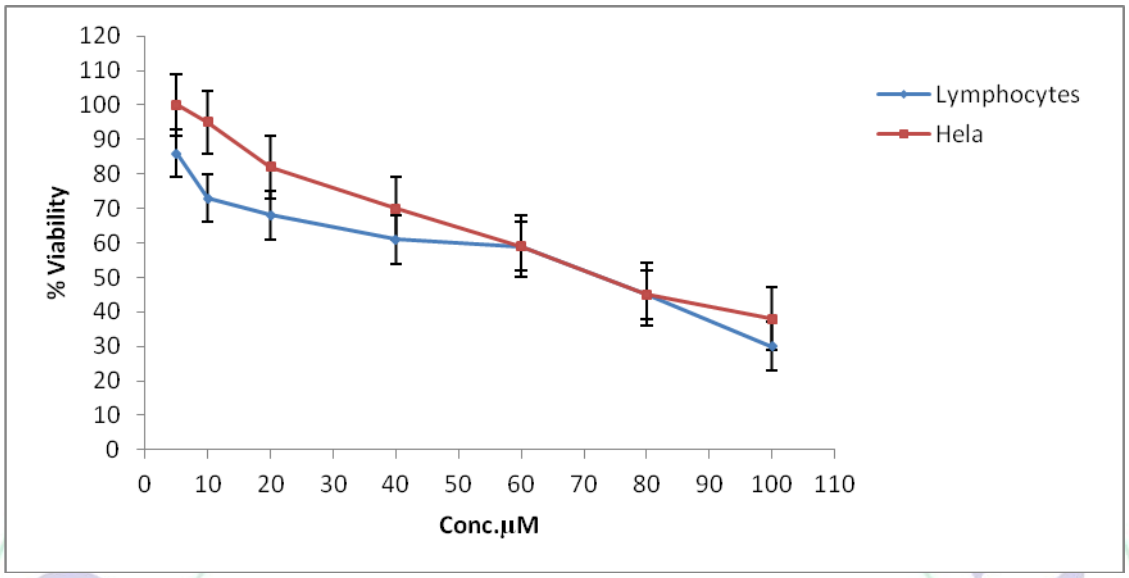


Figure 3: Dose dependent curve of lymphocytes (controls) and HeLa cells after treatment with Vinorelbine at 24hrs. The LC-50 was 78µM for controls and 48µM for HeLa cells.

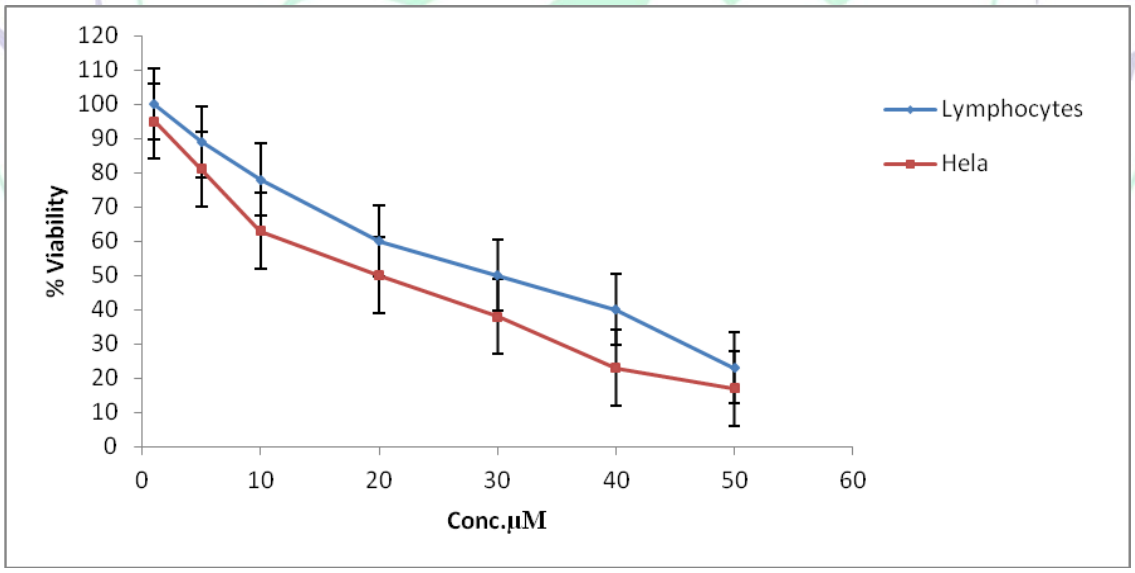


Figure 4: Dose dependent curve of lymphocytes (controls) and HeLa cells after treatment with gefitinib at 24hrs. The LC-50 was 30µM for controls and 20µM for HeLa cells.

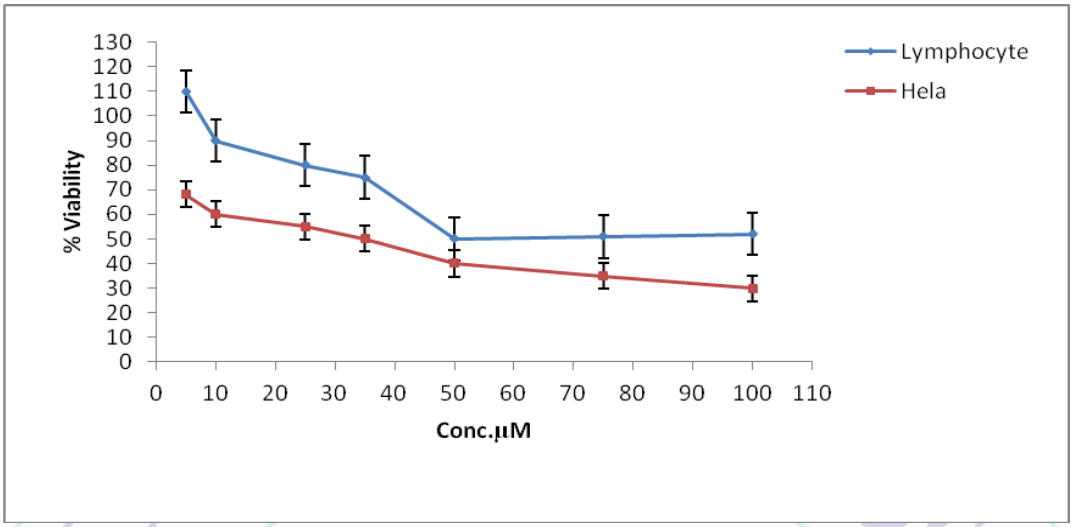


Figure 5: Dose dependent curve of lymphocyte (controls) and HeLa cells after treatment with Gemcitabine after 24hrs. The LC-50 for lymphocytes was 50 µM and 35 µM for HeLa cells.

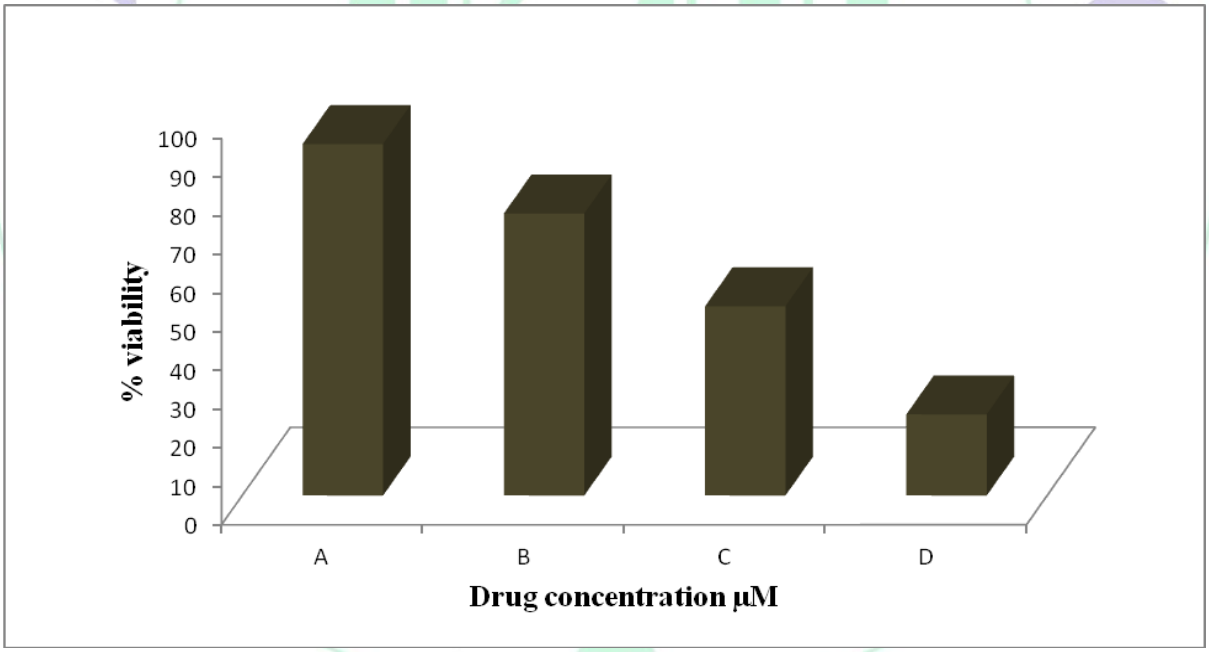


Figure 6: Showing HeLa cell viability after treatment with combination of neoplastic drugs: 5-FU, Cisplatin, Gefitinib at 24 hrs.

A (0.5 µM 5-Fluorouracil + 1µM Cisplatin + 1µM Gefitinib)
B (1 µM 5-Fluorouracil + 1.5 µM Cisplatin + 2 µM Gefitinib)
C (2.5 µM 5-Fluorouracil + 3 µM Cisplatin + 3 µM Gefitinib)
D (4 µM 5-Fluorouracil + 4.5 µM Cisplatin + 4 µM Gefitinib)

Comet Assay (Single Cell Gel Electrophoresis)
The results obtained by comet assay indicated the extent of DNA damage. By treating the lymphocytes with LC-50 concentration of drugs the extent of DNA damage was determined. The measurement of comet tail lengths is given in Table-1. The comet tail length was greater in cisplatin treatments and least with vinorelbine. We found more DNA strand breaks (longest tail length) with cisplatin which was 50.12 ± 4.174µM at 20µM concentration and least damage of DNA with Vinorelbine as shown by the measurement of tail length (33.75 ± 4.91µM) at 78µM.

The DNA damage studies by measuring the comet tail lengths are presented in table-2 for HeLa cells. We found greater DNA strand breaks with Cisplatin treatment (110.65 ± 5.340µM) at 13µM and the least with Vinorelbine (73.85 ± 4.757µM) at 48µM concentration. The order of DNA damage was similar to the order of cytotoxicity (Cisplatin> Gefitinib>Gemcitabine>5-FU>Vinorelbine).

Table 1: Drug concentration vs DNA damage - measuring mean tail length (Comet Assay).

Drug	LC50 Values	Tail length of Comet (µM) Mean ± SEM Lymphocytes
Control	-	3.51±0.739
Cisplatin	20 µM	50.12±4.174**
Gefitinib	30 µM	41.18±1.887***
Gemcitabine	50mM	39.21±3.995**
5-Florouracil	59 µM	37.15±5.456*
Vinorelbine	78 µM	33.75±4.91*

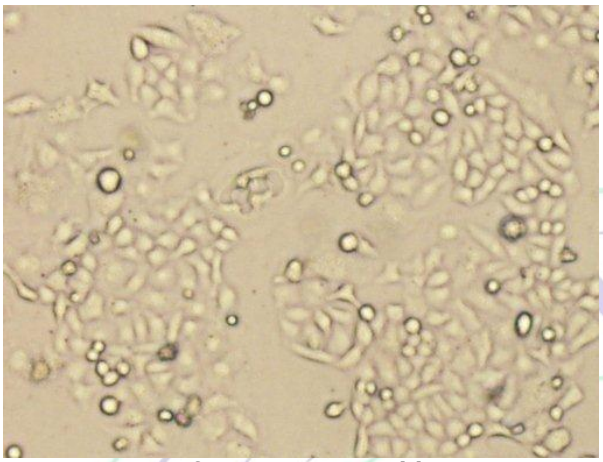
*P<0.01, **P<0.001, ***P<0.0001

Table 2: Drug concentration vs DNA damage - measuring mean tail length (Comet Assay).

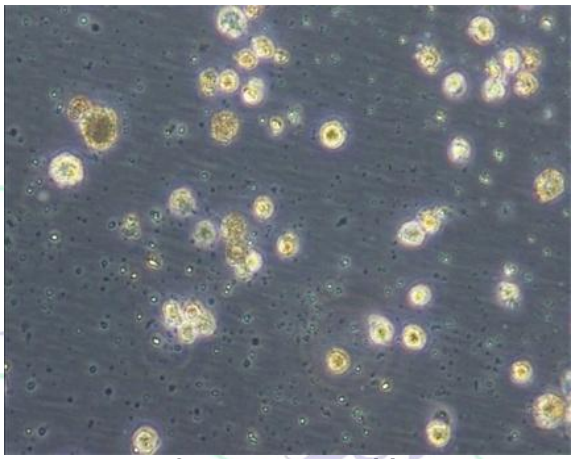
Drug	LC50 Values	Tail length of Comet (µM) Mean ± SEM HeLa cells
Control	-	8.12±0.675
Cisplatin	13 µM	110.65±5.340***
Gefitinib	20 µM	97.15±4.110***
Gemcitabine	35mM	85.65±3.146***
5-Florouracil	40 µM	79.21±3.550***
Vinorelbine	78 µM	73.85±4.757**

*P<0.01, **P<0.001, ***P<0.0001
Data presented are mean results from three independent experiments ± SEM

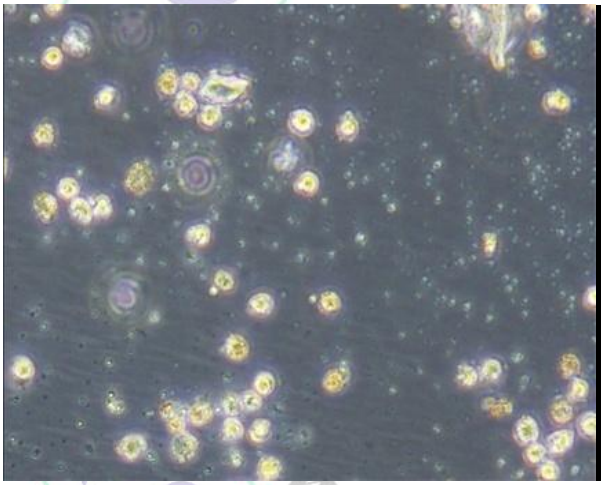
Photos showing morphological changes due to treatment in HeLa cells



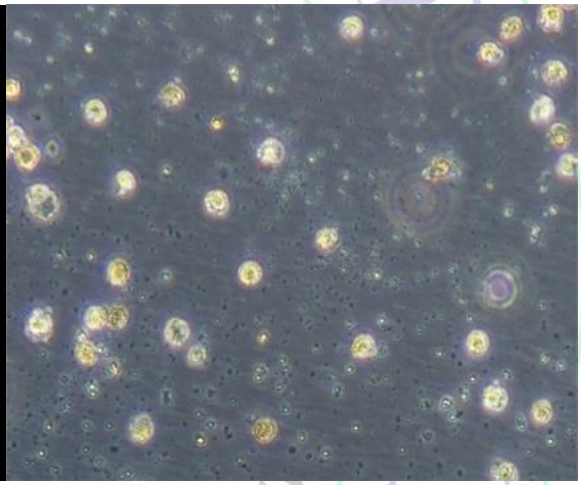
A: Before treatment with 5-FU



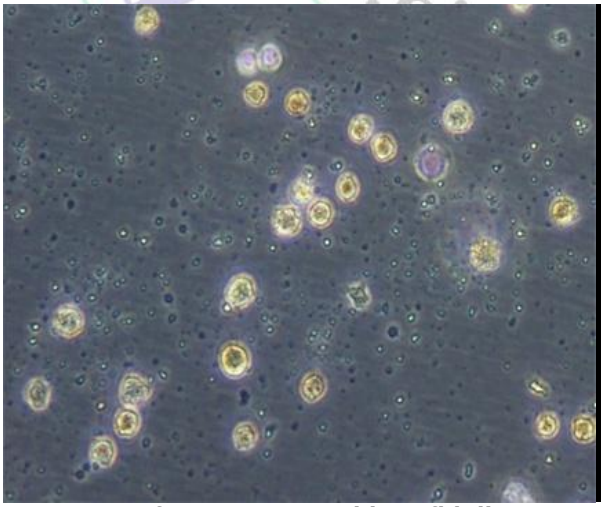
B: After treatment with 5-FU



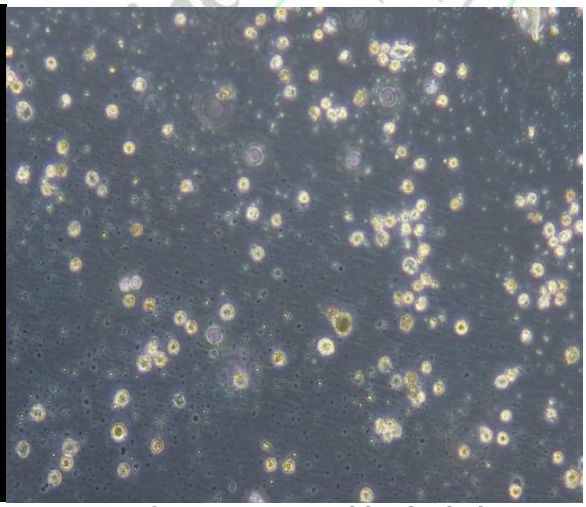
C: After treatment with Gemcitabine



D: After treatment with Vinorelbine



E: After treatment with Gefitinib



F: After treatment with Cisplatin

Discussion

Owing to an improvement in overall survival for cervical carcinoma, recurrent or metastatic disease remains incurable, and chemotherapy is only palliative at this stage. Hence, in our investigation we tried to determine the toxicity of some neoplastic drugs commonly used in palliative care for cervical carcinoma. In order to determine the toxic effects of the neoplastic agents we used *in vitro* technologies, which is an evolving science and has a potential to revolutionize drug toxicity to determine the tolerable and threshold levels of chemotherapy. *In vitro* technologies have been used to determine various end points like DNA damage, cytotoxicity and chromosomal aberration frequencies (Jamil *et al.*, 2004, 2005; Naravaneni and Jamil, 2005a,b; Shaik *et al.*, 2005; Suman and Jamil, 2006a). We have used gefitinib, cisplatin, 5-FU, gemcitabine and vinorelbine singly and in combination to determine their effective doses and their threshold limits. Data from *in vitro* studies have shown that in addition to reducing proliferation in transformed cells, Gefitinib was investigated in a phase II clinical trial as second and third-line single agent for recurrent squamous or adenocarcinoma of the cervix. Gefitinib induces cell cycle arrest, increases apoptosis and has anti-angiogenic activity (Sgambato *et al.*, 2004; Ciardiello *et al.*, 2001; Tortora *et al.*, 2001). In addition, in preclinical models, gefitinib has been shown to have anti-metastatic properties in the following human tumour types: head and neck, prostate, breast, ovarian, colon, small-cell lung and NSCLC (Ciardiello *et al.*, 2001; Ciardiello *et al.*, 2000; Mandal *et al.*, 2002; Sirotnak *et al.*, 2000). Goncalves *et al.* (2008) have documented that gefitinib mono-therapy had stable condition in advanced or metastatic cervical cancer patients.

Cisplatin is the most active cytotoxic agent in metastatic and recurrent squamous carcinoma of the cervix (Bonomi *et al.*, 1985). The 5-FU and Cisplatin combination treatment suppresses the growth of cervical cancer cells by synergistic effect with the induction of apoptosis. A number of investigators have examined the combination of 5-FU and Cisplatin and have yielded encouraging results, suggesting that the combined treatment may be a useful approach (Jacobs *et al.*, 1992; Diaz-Rubio *et al.*, 1992; Scanlon *et al.*, 1986). Cisplatin has consistently proven to be the most effective single cytotoxic agent for the treatment of advanced or recurrent cervical cancer (Thigpen *et al.*, 1995). Combination therapies of carboplatin or paclitaxel have been tried on stage IV cervical cancer patients with much success (Seiji Mabuchi *et al.*, 2010). Our results indicate that this combination was highly effective in killing the cancer cells (HeLa cells) as compared to normal cells. 5-FU is widely used in the treatment of cervical, gastrointestinal, breast, and lung cancers. The mechanism of 5-FU is known to be due to its metabolic conversion to 5-fluorouridine-59-triphosphate with subsequent incorporation into RNA, and/or the formation of 5-fluoro-29-deoxyuridine 59-monophosphate, a well-known inhibitor of thymidylate synthetase. Our results are similar to those reported in literature that is 5-FU inhibits cell viability and its mechanism is inhibiting DNA synthesis through this action in cancer cells. 5-FU down regulates Bcl-2 family and induces caspase family (Yim *et al.*, 2004).

Gemcitabine, a widely used chemotherapeutic drug is a ribonucleotide reductase inhibitor and exhibits cell phase specificity, primarily killing cells undergoing DNA synthesis (S-phase) and also blocking the progression of cells through the G1/S phase boundary (Giovannetti *et al.*, 2004). Our results also reflect not only toxicity but also the dose at which they can control the cell viability. The cellular effects of gemcitabine are maximally exerted on cells in the S-phase of the cell cycle by virtue of its DNA synthesis inhibitory effects (Plunkett *et al.*, 1995). Results of the current study are in agreement with the findings of Dueñas-González *et al.* (2001). Brown *et al.* (2010) have reported that gemcitabine and cisplatin combination response rate was 50% in woman with advanced or recurrent endometrial cancer. Govanneti *et al.* (2006) have reported in-vitro studies on gemcitabine in combination with cisplatin/premetrexed or taxane and showed that it had an enhanced effect on several human cancers both in-vitro and in-vivo.

Further, our results on Vinorelbine demonstrated cell viability inhibition in a dose dependent manner. Vinorelbine is a new generation drug obtained from vinca alkaloids, which exerts its biological effects by inhibiting microtubule assembly (Leveque *et al.*, 1996). It is an active agent against several human malignancies (Harousseau *et al.*, 1997; Monfardini *et al.*, 2001). Cornelio *et al.* (2009) have reviewed in depth, the emerging trends in newer chemotherapeutics for cervical cancer. Their focus has been in describing molecular events in targeted therapies using anti EGFR

drugs/anti VEGF drugs specially agents include interleukin-2, 5-FU, methotrexate, vinblastine, doxorubicin, carboplatin, Taxol, cisplatin and Vinorelbine. Hence, it is possible that Vinorelbine could be an effective drug if it is given in combination with other regular chemotherapeutic drugs.

Other researchers have also reported a comparative study of single drug versus a combination of drugs in cancer cell lines and correlated their sensitivity (Serova *et al.*, 2011). Traditional cytotoxic anticancer therapies do not differentiate between tumour and host cells, and research efforts have been focused on finding new agents that target tumour tissue. To know the effect of individual drugs on DNA damage in both Lymphocytes and HeLa cells, we performed Comet Assay. Results obtained (Table 1 and 2) reflect that among all the 5 studied drugs, Cisplatin showed highest toxicity (long tail formation) at lower doses which is statistically highly significant whereas vinorelbine showed lesser toxicity comparatively (minimal tail formation), which is statistically less significant. Hence, majority of anticancer drugs available may work through a mechanism causing sufficient damage to trigger so called programmed cell death or apoptosis (Hickman, 1992; Zunino *et al.*, 1997). The results of our study were in agreement with other studies (Aydemir, 2005; Kopjar *et al.*, 2006; McKenna *et al.*, 2008).

Treatments for invasive cervical cancer often make it impossible for women to become pregnant in the future, hence for many women - especially younger women and those who have yet to begin a family - infertility is a distressing side effect of treatment. Therefore, our studies have been to evaluate the toxicological end points of these neoplastic drugs, both individually and in combination, which could be useful to the medical oncologist to determine the doses during treatment.

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