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Application of Human Lymphocytes for Evaluating Toxicity of Anti-Cancer Drugs

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Abstract: Important discoveries came forth when human cell lines were used for screening drugs and other chemicals. This investigation determines the toxicity profiles of neoplastic drugs utilizing human lymphocytes from peripheral blood samples of healthy non-smoking donors and the blood samples of cancer patients suffering from acute myeloid leukemia (AML) were collected. The order of toxicities (LC50) using MTT assay was found to be as follows; Cisplatin > Carboplatin> Cyclophosphamide > 5-FU > Vincristine. The values were calculated using statistical analysis. The MTT assay is now widely adopted by researchers and industry, as it is a rapid Spectrophotometric method for determining cell viability in cells. The changes induced by individual drugs could be unique to each compound which might reflect in genotoxicity, hence utilizing the Single Cell Gel Electrophoresis (SCGE) assay, genotoxicity of the drugs was determined as comet tails of DNA damages. It was found that the values obtained were significant ($p<0.04$) and showed that the damage was highest in cisplatin as compared to other drugs. The order of toxicity in comet assay was similar to that of MTT assay. It is therefore concluded that the two assays could be used simultaneously to determine the toxic properties of the drugs on human cells and can be compared to cancer cells.

Key words: Anti-cancer drugs, comet assay, cytotoxicity, genotoxicity, *in vitro* assays, MTT assay

INTRODUCTION

Anti-tumor activity of various drugs is based on the mechanism of action of how these agents are effective in killing the tumor cells or in preventing the growth of the tumor cells. There are several methodologies which aim to arrive at these results and correlate these drug effects in patients, so as to assist the medical oncologist in selecting the most appropriate drugs to be used in the treatment of the individual patients. Tumor cells unlike normal cells fail to respond to homeostatic control mechanisms, as a result their population expands due to continuous cell division (Schabel, 1975). Cell kinetic techniques have allowed an understanding of tumor growth (proliferation) in relation to normal cell growth (proliferation) (Tannock, 1992). Hence the fundamental kinetic consideration in cancer chemotherapy is "cell-kill" hypothesis, derived from detailed studies in animal tumor models (Clark and Kamen, 1987).

There is a common belief that anti-cancer drugs produce non-selective cell killing of normal as well as cancerous tissues. Although this belief is somewhat true for certain agents such as Mechlorethamine (mustargen, HN_2) and carmustine, the majority of anti-cancer drugs act more against tumor than normal cells. The hormonal agents are best examples of anti-cancer agents, but these agents tend to produce more toxic effects on normal bone marrow cells (Devita *et al*., 1975). Therefore much research needs to be done in the search for drugs that can exploit the unique biochemical difference between normal and neoplastic cells. It is believed that normal systems can theoretically withstand greater cellular loses to chemotherapy agents than can tumor cells (Hampton, 2004). Thus application of normal and cancer cells have been used in this investigation.

Cisplatin, carboplatin and cyclophosphamide, belong to alkylating agents that act through the covalent bonding of alkyl groups to intracellular macromolecules. The mechanism of cancer cell toxicity is due to the binding of the alkyl groups directly to DNA bases. This result in DNA damage in the form of single-strand breaks and cross-linking which ultimately prevents the cell from reproducing. 5-FU is an anti-metabolite and an analog of a normal metabolite. Vincristine is an alkaloid obtained from the periwinkle (*Vinca rosea* Linn), *in vitro*, it arrests mitotic cell division at metaphase.

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In vitro technology is an evolving science which has a potential to revolutionize drug toxicity to determine the tolerable and threshold levels. *In vitro* technologies have been used for determining various end points like DNA damage, Cytotoxicity and chromosomal abberation frequencies (Jamil *et al*., 2004, 2005, Naravaneni and Jamil 2005a,b; Shaik *et al*., 2005; Suman and Jamil 2006a,b). Therefore, adopting these *in vitro* technologies, we have carried out investigations to determine the cytotoxicity and genotoxicity of certain neoplastic drugs *in vitro*.

Most of the anti-cancer drugs target the enzyme systems in the cell cycle to block cell division. However there are differences in the mechanism of action, based on differences in the chemistry of the drugs, for example the action of platinum based drugs, is different from the alkylating agents. As early as 1984, Weisenthal *et al*. (1984) have described chemo sensitivity assays on the basis of total kill of the tumor cells *in vitro*, subsequently these methods became widely used in leukemias (Weisenthal, 1993). Toxicity refers to the dosage levels, hence in this investigation we attempt to utilize human cells from normal and cancer patients to determine the comparative toxicity of the drugs. We have selected *in vitro* assays using lymphocytes from normal and leukemic subjects, which could be measured easily by MTT assay (for cytotoxicity) and comet assay (for genotoxicity).

So far only very few reports are available on the comparative toxicity of tumor cells with normal cells. The drugs chosen for his study belong to two different mode of action such as alkylating agents (Cisplatin, carboplatin and cyclophosphamide) and antimetabolites (5-FU and vincristine).

MATERIALS AND METHODS

The anti-cancer drugs used in this study are cisplatin carboplatin, cyclophosphamide, Vincristine and 5-FU.

Cisplatin: IUPAC name: *cis*-diamminedichloroplatinum (II) Molecular formula: $N_2Cl_2PtH_6$, Molecular weight: 300

It was obtained from Cadila Pharmaceuticals Ltd (Platin 50) in aqueous form (50 mg in 50 mL).

Carboplatin: IUPAC name: Diammine (1,1-cyclobutane-dicarboxylato(2-)-*o*, o')-platinum (II)
Molecular formula: $C_6H_6O_4Pt$, Molecular weight: 337.2016

It was obtained from VHB Pharmaceuticals Ltd (Carbotinal) in aqueous form (150 mg/15 mL).

Cyclophosphamide: IUPAC name: 2-(bis (2-chloroethyl) amino) tetrahydro-2H-1, 3, 2-Oxazaphosphorine 2-oxide monohydrate; Molecular formula: $C_7H_{15}N_2O_2P$, Molecular weight: 261.08

It was obtained from Cadila Pharmaceuticals Ltd (Oncophos 1000) in powder form and solubilized in water 1000 mg of drug was dissolved in 50 mL of double distilled water. From this reconstituted solution 1M of Cyclophosphamide drug was prepared and various aliquots from the stock solutions were used throughout the experiment.

5-Fluorouracil (5-FU): IUPAC name: 5-fluoro-2, 4g (1*H*,3*H*) –pyrimidinedione .
Molecular formula: $N_2FO_2H_3$, Molecular weight: 130.08
It was obtained from Dabur Pharmaceuticals Ltd in aqueous form (1 mg/1 mL).

Vincristine: Molecular formula: $C_{47}H_{58}N_4O_{10}$, Molecular weight: 839.0018

It was obtained from Sun pharmaceuticals Ltd (Oncocristin AQ) in aqueous form (1 mg/1 mL).

Preparation of drug solutions: All the drugs were solubilized in water and 1M concentration of all the drugs were prepared by using the molecular weights of each drug. Various aliquots from the stock solutions were used throughout the experiments as indicated.

Collection of blood samples from cases and controls: Fresh blood from healthy non-smoking donors was collected in heparinized syringes and transferred in the Eppendorf tubes and used immediately for the experiments described below.

Short term lymphocyte culture using the whole blood samples were set up following the methods described earlier by us (Jamil *et al*., 2004; Merk and Spelt, 1999). Simplest method of lymphocyte culturing is by incubating of a small amount of whole blood by adding PHA to initiate growth of lymphocytes without previous separation of erythrocytes and granulocytes. This utilizes all available lymphocytes, which are usually 25-50% of the leukocyte count i.e., 1,800-5,000 cells mm^{-3} of blood. With the informed consent, 3 mL blood was collected from 5 AML patients after Institutional Ethical Committee clearance. Blood was collected from the patients prior to the chemotherapy in heparinized syringes and transferred in the Eppendorf tubes and used for isolating the lymphocytes for the experiments as described.

Experimental protocol: Pure lymphocytes were isolated from the blood samples by the Ficoll density gradient technique (Boyum, 1968) for MTT assay and blood samples were used directly for comet assay. Sub-lethal concentrations of the drugs were used for *in vitro* experiments. Lymphocytes and blood samples were

treated with the various aliquots of each drug for two hours and the experiments as described below were carried out, in triplicates and averages recorded. The experiments were carried out at Bhagawan Mahavir Medical Research Center, Hyderabad, India during 2004-2005.

MTT Assay for determining Cytotoxicity: The MTT assay for cell viability was used to determine the lethal concentration of the drugs as described earlier by us (Suman and Jamil, 2006a,b). An increase or decrease in cell number results in a concomitant change in the amount of formazan formed, indicating the degree of cytotoxicity caused by the test material (Cartwright *et al*., 1997; Dash *et al*., 2003).

Growth inhibition is expressed as;

$$T/C\% = \frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \times 100$$

Where, T = Total: C = Cell count

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

Statistical methods: The data obtained in this investigation was subjected to statistical analysis, using simple analysis of variance (ANOVA) and mean and Standard Deviation (SD) was calculated to show the central value of the observed data with deviation and to prove the percent of variation in experimental observation, Coefficient of variation was calculated. All these statistical analysis were applied to determine significance of the results between treated and untreated samples.

RESULTS

Comparative toxicity of the drugs in normal blood samples: Relative cytotoxicity of Cisplatin, Carboplatin, Cyclophosphamide 5-Fluorouracil and Vincristine carried out by MTT assay were compared and the results presented in Table 1 and Fig. (1-5) for each drug. The survival curves indicated that carboplatin was 2.5 times less effective in killing the cells compared to cisplatin. In other case of drugs , survival curves indicated that cyclophosphamide was nearly 2 times less effective than carboplatin; 5-FU was nearly 27 times less effective than cyclophosphamide; and vincristine was nearly 3 times less effective in killing the cells when compared to 5-FU (Fig. 1-5).

Table 1: MTT assay showing the viability of lymphocytes treated with various drugs

Drug	Concentration Used (nM)	Lymphocyte concentration (µL)*	OD at 570 nm	Viability as calculated (%)
Control (Untreated lymphocytes)	-	100	0.923	99
Cisplatin	0.02	100	0.563	59.60
	0.04	100	0.512	55.79
	0.06	100	0.459	49.68
	0.08	100	0.392	41.34
	0.10	100	0.354	35.63
	0.12	100	0.322	31.56
	0.14	100	0.298	27.39
Carboplatin	0.10	100	0.592	66.12
	0.12	100	0.573	60.22
	0.14	100	0.483	54.063
	0.16	100	0.456	49.46
	0.18	100	0.412	45.33
	0.20	100	0.389	41.02
	0.22	100	0.345	36.44
Cyclophosph-amida	0.16	100	0.572	60.03
	0.20	100	0.522	56.98
	0.24	100	0.486	53.63
	0.28	100	0.461	49.79
	0.32	100	0.409	44.78
	0.36	100	0.372	39.99
	0.40	100	0.342	35.65
5-FU	4.5	100	0.582	62.22
	5.5	100	0.511	56.284
	6.5	100	0.487	53.641
	7.5	100	0.461	49.5
	8.5	100	0.396	43.32
	9.5	100	0.367	39.76
	10.5	100	0.319	30.12
Vincristine	5.0	100	0.592	62.64
	10.0	100	0.513	56.013
	15.0	100	0.481	53.11
	20.0	100	0.461	49.97
	25.0	100	0.398	42.09
	30.0	100	0.339	34.32
	35.0	100	0.289	28.72

* Lymphocyte concentration is approximately 50,000 cells/100 µL in each sample

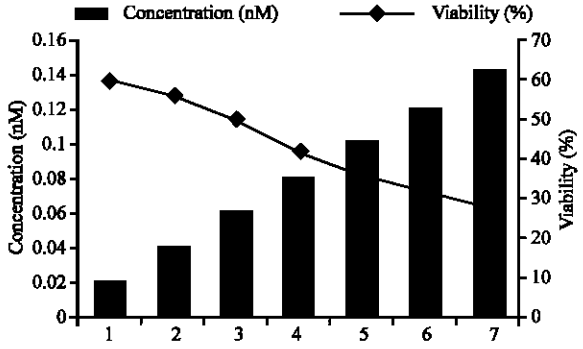

Fig. 1: Showing the % viability vs conc. of drug (Cisplatin)

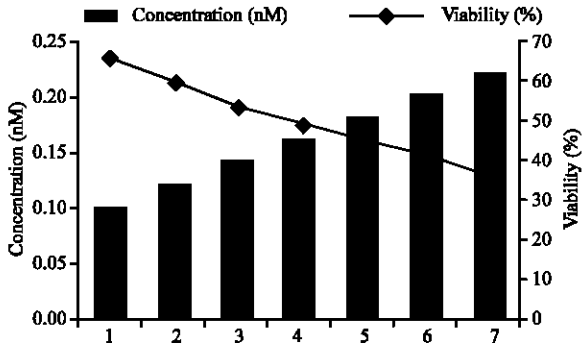

Fig. 2: Showing the % viability vs conc. of drug (Carboplatin)

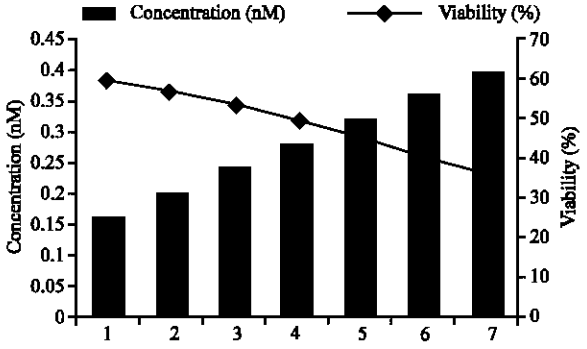

Fig. 3: Showing the % viability vs conc. of drug (Cyclophosphamide)

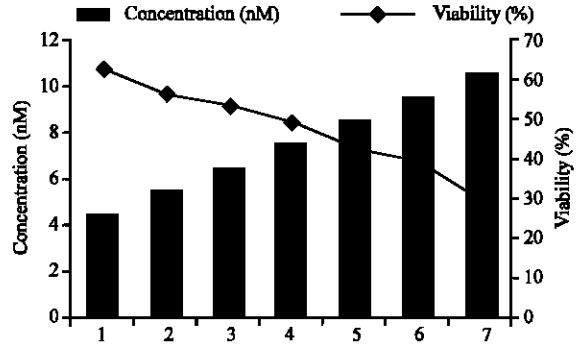

Fig. 4: Showing the % viability vs conc. of drug (5-Fluorouracil)

These results may reflect the functional similarity of the two platinum compounds which were found to be highly toxic drugs (Table 1 and Fig. 1, 2).

Comparative toxicity of drugs in lymphocytes of cancer patients (AML Cases): Relative cytotoxicity of cisplatin, carboplatin, cyclophosphamide 5-fluorouracil and

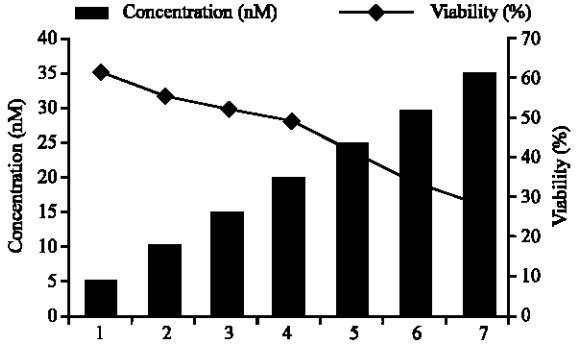

Fig. 5: Showing the % viability vs conc. of drug (Vincristine)

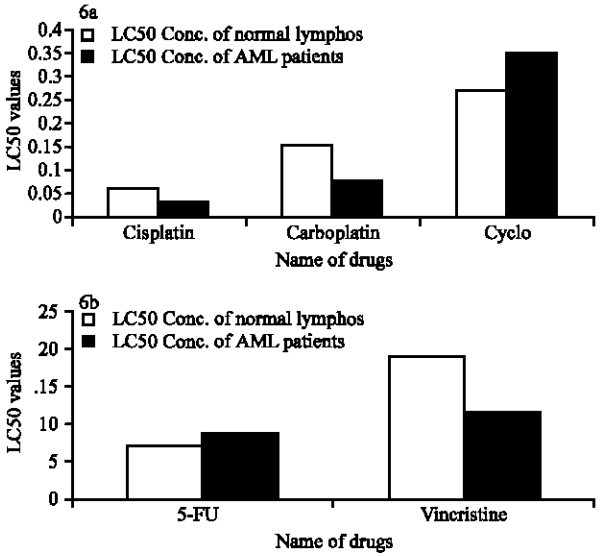

Fig. 6: Comparative LC50 values of the drugs in normal and AML lymphocytes

vincristine carried out by MTT assay were compared in leukemic cells and the results presented in Fig. 6 (a,b). The cell viability was much less as compared to normal cells. The three drugs cisplatin, carboplatin and vincristine were highly toxic to leukemic cells as compared to normal cells. Whereas cyclophosphamide and 5-FU were equally or slightly more toxic to normal cells as compared to leukemic cells.

Calculating the LC50 values of the drugs: By substituting the values of drug concentrations and the ODs obtained in the given MTT formula, the percentage of lymphocyte viability was calculated, as per the method given above. The drug concentration at which half of the control OD obtained is termed as LC50 concentration. In this investigation the nanomolar concentration (LC50) for five anti-cancer drugs (cisplatin, carboplatin,

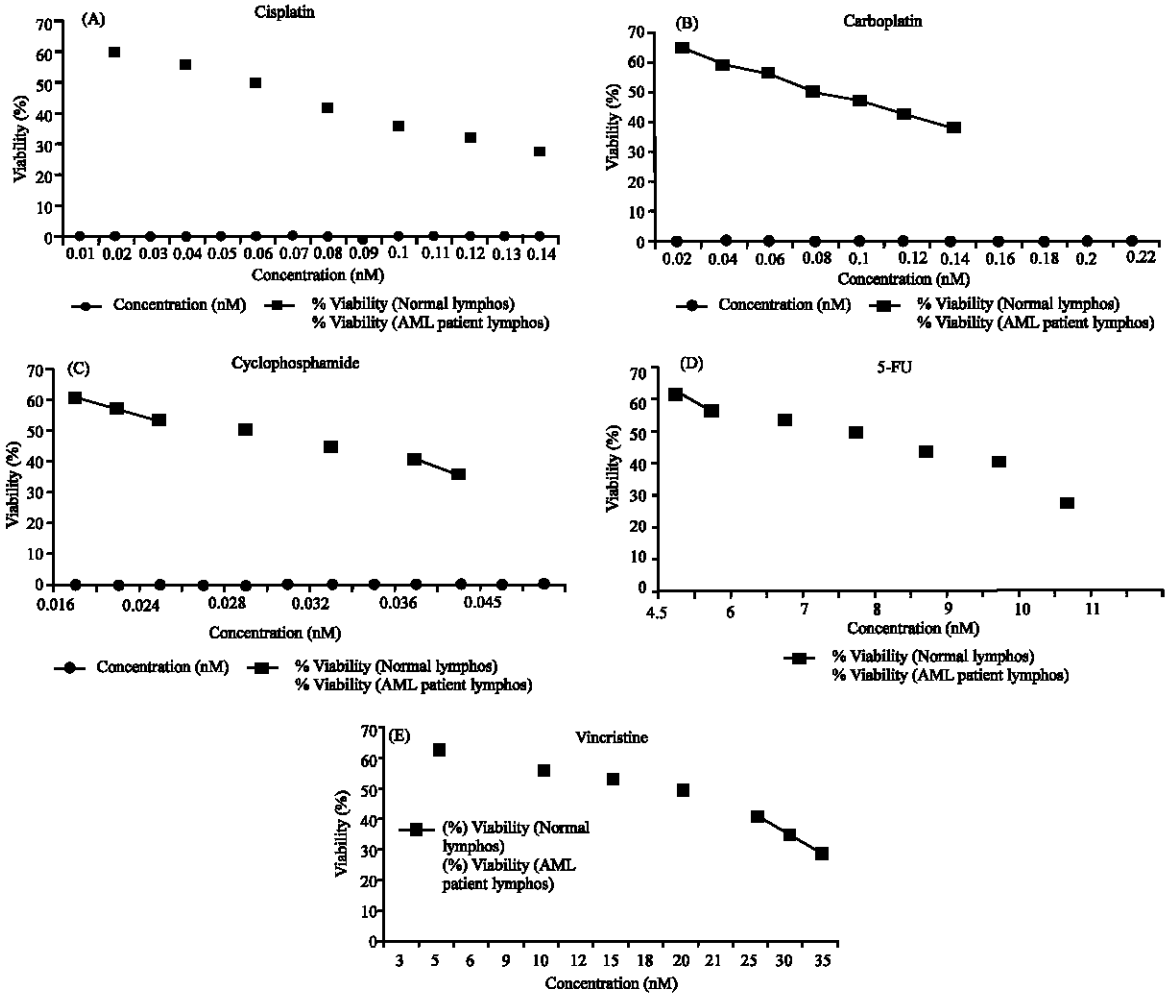

Fig. 7: Comparative viability of lymphocytes in normal and acute myeloid leukemia (AML) Patients

cyclophosphamide, 5-FU and vincristine) was found to be 0.06, 0.16, 0.28, 7.5 and 20.00 nM in that order in normal cells and Cisplatin-0.03 nM, Carboplatin-0.08 nM, cyclophosphamide-0.35 nM, 5-FU-9 nM and vincristine-12 nM in leukemic cells (Fig. 6a, b). Comparative viability of lymphocytes in normal and acute myeloid leukemia (AML) patients is shown for drugs individually (Fig. 7a-e).

DNA damage studies (On normal cells): The results obtained by comet assay (single cell gel electrophoresis) indicate the extent of DNA damage. By treating the blood samples with LC50 concentration of the drugs, the extent of DNA damage in lymphocytes was determined. The measurement of the comet tail lengths is given in Table 2 and Fig. 8. The order of DNA damage was similar to the order of cytotoxicity (cisplatin > carboplatin > cyclophosphamide > 5-FU > vincristine). It is seen that

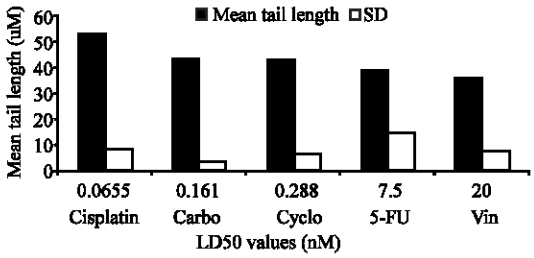

Fig. 8: Comparative toxicity of drugs in comet assay: LD50 values of the drugs along with Standard deviations (SD) and Mean tail length (μm)

the comet tail length was greater in cisplatin and least in vincristine. The longest tail length we observed was (52.4294±8.238 μm) which was at a 0.0655 nM concentration of cisplatin and the least tail length we observed was (35.8655±7.062 μm) which was at a 20 nM concentration of vincristine.

Table 2: Drug concentration vs mean comet tail length

Drug	LC_{50} values (nM)	Tail length of comet (μm) (Mean±SD)	Coefficient of variation
Control	-	3.299±1.171	0.354956047
Cisplatin	0.0655	52.4294±8.238	0.157125582
Carboplatin	0.161	42.5584±3.114	0.073170044
Cyclophosphamide	0.288	42.2627±6.681	0.158082659
5-FU	7.5	38.4491±14.344	0.217014182
Vincristine	20.0	35.8655±7.062	0.196902316

Statistical analysis: Coefficient of variation was calculated and presented in Table 2; it shows the possible variations in observation of these 5 anti-cancer drugs. The coefficient of variation shows the comparative accuracy of the data with respect to each drug tested (Fig. 8 and Table 2). It was found that cisplatin was more toxic than other drugs according to the experimental observations and also as seen by these statistical calculations. Figure (1-5) indicate that the concentration of the drugs is inversely proportional to the viability of the cell populations in all cases. While comet pictures as observed in the microscope also indicate similar results as represented by the comet tail lengths verses the concentration of the drugs.

DISCUSSION

The tests used can determine not only the toxicity but also the dosage levels at which these can produce the toxicity. All the anti-cancer drugs are highly cytotoxic agents and may be toxic to normal cells specially to rapidly dividing cells like bone marrow cells, fetal cells, germ cells, hair follicle cells, intestinal cells, etc. The kinds of side effects they produce are well known (Kopjar *et al*., 2002). In order to evaluate the cytotoxicity and genotoxicity of the drugs we have used simple techniques like MTT assay and comet assay to determine the mortality of cells (normal and leukemic) *in vitro*. It is seen from our results that the order of toxicity remained the same, but the concentrations required for normal cells was much higher than that required for leukemic cells. The order of toxicity was as follows;

Cisplatin > carboplatin > cyclophosphamide > 5-FU > vincristine

These assays can be used to identify the best dosage levels on each individual basis. Generally chemotherapy has been identified in controlled clinical trails, thus these assays could become part of such trails. However in acute myeloid leukemia vincristine and carboplatin are generally used, whereas cisplatin is used in therapy related myelodysplasia and 5-FU is given as adjuvant therapy and cyclophosphamide is generally recommended in relapsed cases. With wide spread use of the *in vitro* assays in clinical laboratory, it is more likely that the activity of new drugs and new regimens would be identified in a shorter time period than with the current system relying exclusively on-empiric phase II trials (Weisenthal, 1992). It may be true that the effect of chemotherapeutic agents on tumor cells can be more than ten thousand times greater than that on normal cells (Weiss *et al*., 1987). In our study we found higher toxicity on cancer cells with 3 drugs. This difference can be attributed to the biochemical differences between normal cells and cancer cells. Much research can to be done in the search for the drugs that can exploit the unique biochemical differences between normal and neoplastic cells. Also, tumor drug selectivity may still be based largely on differences in the cell kinetics of normal and neoplastic cells. For this reason we have calculated the coefficient of variation to find out the relative toxicities of the five drugs tested. From these results, it is seen that these two assays have good correlation to assess the cytotoxicity and genotoxicity of various anti-cancer drugs to predict responses at cellular levels *in vitro*.

Normal cell systems can theoretically withstand greater cellular losses to chemotherapy than can tumors (Boileau *et al*., 1971). The *in vitro* assays use various end points like MTT, ATP, DNA strand breaks, etc., for measuring cell kill, or for understanding the mechanism of action of the drugs. Only when these assays are widely used and routinely included as an integral part of clinical trails, their role as 'promising technologies' could be evaluated. Single strand breaks were easily recorded using comet assay for the five drugs tested, but the tail lengths varied with each drug that was tested, giving the indication of the varied effects of DNA damage with each drug.

The coefficient of variation data also shows the concentration of drug and corresponding mean tail length of comet. SD of tail length is the measure of dispersion which provide the range (from mean-SD to mean + SD) of tail length observation. There is evidence that the majority of available anti-cancer drugs may work through a mechanism of causing sufficient damage to trigger so called programmed cell death or apoptosis (Hickman, 1992; Zunino *et al*., 1997). Since all the drugs used in this study interact directly or indirectly with DNA, this investigation determines the dosages at which these drugs can bring about DNA strand breaks (genotoxicity) and degree of cytotoxicity (MTT assay). The percent lethality of drugs tested on cell cultures serves a measuring index of drug toxicity.

Critical assessment: Chemotherapy works because it kills cancer cells. Unfortunately, the drugs can also kill healthy cells as they pass through the body on their way to the cancerous target. Many are so toxic so they never make it to the market. Those that make it to the market are not free of side effects or adverse effects, or even end up with being ineffective (resistant).

The adverse effects may be due to the fact that the quantity or dosage of these drugs circulating in the body could be triggering these effects. It was found that some drugs are used in large amounts technically than others to produce the same effect i.e., tumor cells-kill. The effects of platinum compounds (Cisplatin and Carboplatin) are milder because the quantity circulating in the blood is much less when compared to the alkylating agents as per our cytotoxic index (Fig. 6a, b).

This is just the beginning of a new hypothesis; many studies like these may give us useful answers. The knowledge gained by these assays, could be useful to the clinicians to taken into account the dose levels to be prescribed.

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