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# Characterising cytotoxicity and genotoxicity of chemotherapeutic agents (Etoposide and Vincristine) on TK6, a human lymphoblastoid cell line

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## Abstract

The ability of the chemotherapeutic agents, namely etoposide and vincristine, to cause cytotoxic and genotoxic damage to the human lymphoblastoid cell line, TK6, was at the centre of this study. Four different cytotoxic assays were tested for their ability to assess the cytotoxicity of the agents as well as two genotoxic assays to examine their ability to induce DNA damage. The TK6 cell line was exposed to increasing doses of the chemotherapeutic agents and cytotoxicity assessed by *in vitro* trypan blue, neutral red uptake, LDH leakage and the glucose oxidase assays. The trypan blue assay was deemed to be the most beneficial due to its ease and speed of quantifying the number of non-viable cells as through the loss of their membrane integrity they stain the characteristic blue colour. Genotoxicity on the other hand was measured by two assays; the micronucleus and Comet assays. Both are currently used within the laboratory setting but this study concluded the Comet assay was the best method for measuring DNA damage. Additionally, vincristine was found to be more cytotoxic than etoposide and also exerted further genotoxic effects upon the cells. Although only a pilot study, this work aimed to define the best cytotoxic and genotoxic detection methods using two widely used chemotherapeutic agents and show how they can impact upon cells and cancer/leukemic treatment regimens.

## **Introduction**

Extensive proliferation in mesenchymal and pluripotent stem cells is cited as the cause of haematological malignancies. Leukaemia accounts for 2.4% of all cancers in Europe; in 2008 there were 78,416 reported cases of lymphoid and/or myeloid forms of the disease (Visser *et al.*, 2012).

Chemotherapy has become a key intervention in the treatment of cancer and leukaemia. Generally, its use facilitates in the blocking or slowing down of cell growth. It works by targeting the cell cycle so any cell, diseased or healthy, in cell cycle at the time of administration will be affected. It is this which causes the side effects commonly associated with this form of intervention because the effects are seen where there is evidence of rapid cell proliferation such as the lining of GI tract and hair follicles hence hair loss, nausea and vomiting (Tolmack Sugeran, 2013). The use of haemopoietic stem cell transplantation is regarded as a possible life saving intervention for such diseases. However, the treatment requires the patient to undergo conditioning regimens which achieve total body irradiation thus reducing the risk of graft vs host disease and the activity of the malignancy itself (Gyurkcoza and Sandmaier, 2014). High dose chemotherapy regimens are myelosuppressive in that they irradiate the immune system to reduce the risk of relapse. However, although successful, such regimens display weaknesses such as increased risk of secondary malignancy. Noxious effects on the gastrointestinal, hepatic and pulmonary systems often result in death and in the case of children can affect growth and development (Gyurkcoza and Sandmaier, 2014). The increased toxicity associated with this form of regimen deems it inappropriate in the treatment of haematological malignancies in elderly patients. This is a fundamental issue as highlighted in a study by Puig *et al.*, (2011) who stated that 20-44% of patients diagnosed with Hodgkin's Lymphoma were above the age of 60. Hence there is a need for a regimen which is not myeloablative, but could provide enough immunosuppression for successful transplantation in patients who are unable to tolerate the standard treatment (Bornhäuser *et al.*, 2012).

The use of reduced intensity conditioning (RIC) has had a fundamental impact upon the treatment of leukaemia, particularly for the use of allogeneic haemopoietic stem cell transplantation (Bornhäuser *et al.*, 2012). Its use has allowed for the potential of allogeneic stem cell transplantation in elder patients and not just exclusively for younger patients, therefore becoming a curative method in the treatment of haematological cancers in patients who may have organ damage/dysfunction or who may have experienced complications during initial treatment as a result of infection (Bornhäuser *et al.*, 2012). It is well documented that the transplantation of allogeneic stem cells is the gold standard in the treatment of many haematological diseases. However, due to the related toxicities of using high dose chemotherapy this was restricted to patients who were considered to be medically fit; those who were largely below the age of 55 (Schmid *et al.*, 2006). That was until the introduction of reduced intensity conditioning which was not reliant on the patient being exposed to high doses and thus treatment related toxicities (Schmid *et al.*, 2006). The risk of chemotherapy-induced toxicity increases with the presence of comorbidities linked to increasing age as well as physiological changes occurring within the body (Shayne *et al.*, 2007). The foundation for using RIC was to give a therapeutic regimen which provides adequate immunosuppression in the patient in order to attain engraftment of donor cells, allowing these cells to recover haematopoietically and finally to

achieve a graft versus leukemic status (Ringdén *et al.*, 2013). However, relapses of the disease are the most common cause of failure of stem cell transplant which accounts for around 33% of deaths after transplantation (Barrett, 2008). Although many of the side effects of chemotherapy such as hair loss, nausea and vomiting are expected and also reversible, patients are facing more long term complications such as secondary malignancies (Morgan and Rubin, 1998). This was also true in the Intergroupe Francophone du Myélome trial which was ended in 2011 due to patients with multiple myeloma who were treated with Lenalidomide had a higher significance of secondary malignancies compared to patients treated with a placebo (Fenk *et al.*, 2012). Alkylating agents and topoisomerase II inhibitors are known to increase the susceptibility of secondary malignancies. Leukaemia arising from treatment with alkylating agents tends to have a prolonged period of inactivity of around five years. However, patients exposed to topoisomerase II inhibitors can have a latency period which is significantly lower, approximately two years and the leukaemia can demonstrate cytogenetic abnormalities (Seiter *et al.*, 2001). The incidence of secondary leukaemia is compared to the general population. Therefore, as these patients have already had exposure to a previous malignancy the incidence will be increased particularly as the general population won't have had any previous exposure (Hasegawa *et al.*, 2005). A study by Hasegawa *et al.*, (2005) corroborates that recipients of bone marrow transplants are at a greater risk of secondary malignancy and this association is not reduced over time. The peak occurrence of a secondary malignancy following bone marrow transplant with a total body irradiation chemotherapy regimen is seven years however even after a median follow up of 11 years the risk was still high. As well as this the majority of secondary malignancy occurred within the older population verifying age as a predisposing factor (Hasegawa *et al.*, 2005).

Generally chemotherapeutic agents are given in combinations in order to exploit their effects synergistically. This can be achieved by including chemotherapeutic agents which have distinct modes of action within the combination. This therefore poses challenges in the assessment of individual agents and their therapeutic effects. However, a study by May *et al* (2012) looked at the toxicity of chemotherapeutic agents and their damage to mesenchymal stem cells (MSC) as well developing a suitable *in vitro* model to assess the agent's toxicology. The novel *in vitro* model devised by May *et al.*, (2012) allows the cytotoxicity of a chemotherapeutic agent to be analysed on a different type of cell i.e. MSC. Moreover, the models resemblance of the *in vitro* situation is useful particularly as the limitations experienced when using animal models such as misjudging the toxic effect of a specific agent due to inter-species differences can mean potentially effective drugs are disregarded for further testing as a result of the effect they have on an animal model (May *et al*, 2012).

The toxic effects of two chemotherapeutic agents namely etoposide and vincristine are being assessed through the application of four cytotoxicity assays and two genotoxicity assays. The TK6 cells will be exposed to a reducing dose of each agent in all of the assays but the genotoxicity assays will focus on a low dose and a physiologically relevant concentration in comparison to an untreated control.

Vincristine is a vinca alkaloid which is extracted from the plant *Vinca rosea* and has been in use as a cytotoxic agent for the treatment of cancer since 1962. It's still in use today for a variety of haematological and solid malignancies. Its mechanism of action is through binding to tubulin dimers which in turn inhibits the formation of

microtubules and mitotic spindles. Ultimately this leads to apoptosis as mitosis is arrested in metaphase and thus the cells are unable to divide (Moore and Pinkerton, 2009). Vincristine is metabolised in the liver to metabolites with a reduced activity which supports eventual excretion. This metabolism is carried out by the enzymes CYP 3A4 and 3A5 (Moore and Pinkerton, 2009).

Etoposide on the other hand is a topoisomerase inhibitor derived from the plant alkaloid podophyllotoxin. These agents modify the structure of DNA by forming a cleavage complex, it is this DNA break which is taken advantage of in these anti-cancer drugs as it prevents the re-ligation of the DNA strands causing apoptosis (Wu *et al.*, 2011). Type II topoisomerases create a cleavage complex and passage through this and the subsequent resealing alters the shape of DNA. However, if religation fails to occur cell death can result. This is the process which anti-cancer drugs such as etoposide exploit by increasing the number of cleavage complexes and stimulating the development to cytotoxic lesions in the DNA thus promoting cell death (Wu *et al.*, 2011).

The rationale for using four cytotoxicity tests and two genotoxicity assays is due to the fact that each one measures toxicity in different ways. The use of cytotoxic assays has become an important aspect of explaining the toxicity of agents as well as monitoring both harmful and beneficial effects an agent may have on human cells. There are a number of different assays which can be employed to assess cytotoxicity. All of them have their advantages and disadvantages but the aim of this study is to establish the most suitable assay to use in a more extensive study. One of the most common ways of measuring cytotoxicity is through viability assays. The viability assay used in this study was trypan blue. This type of assay is non-specific and is successful in staining dead/non-viable tissues or cells a characteristic dark blue colour. The method by which trypan blue works is the dye itself is negatively charged and only if a particular cells membrane is damaged does it interfere with it. This therefore means that any viable cells will not take up the trypan blue because the membrane of a cell is particular about which substances can pass through. Live cells also have efflux pumps at work which will prevent the dye getting into the cell by pushing out any dye managing to enter the cell (Tran *et al.* 2011). To contrast the trypan blue stain, a neutral red assay was also undertaken. However this is an uptake assay where the red dye is taken up by the lysosomes of undamaged cells, thus the more red dye which is read the more viable cells are present (Kura *et al.*, 2014). As well as this the use of a lactate dehydrogenase assay where the release of intracellular enzymes is measured has been utilised in a number of studies (Ivanova and Uhlig, 2008). This release of proteins/enzymes is known as a leakage assay because when cells are exposed to cytotoxic agents their membranes can lose their integrity causing the release, in this case of LDH into medium surrounding the cells. Therefore, the discharging of LDH into the medium is a sign of cell death as a result of the membranous damage caused by the cytotoxic agents the cell has been exposed to. This assay in particular is renowned for its simple assessment (Fotakis and Timbrell, 2006). The glucose assay is another way of measuring cytotoxicity. The culture medium in this assay includes glucose which is needed for cells to metabolise and develop. There is evidence that leukemic cells have an increase in glucose utilisation in order to maintain energy homeostasis in the cellular environment and resist apoptosis sustaining progression of the disease (Shanmugam *et al.*, 2009).

To test genotoxic effects two different methods have been employed; the comet assay and the micronucleus assay. The comet assay is also known as single-cell gel electrophoresis (SCGE) and is a technique used to detect damage to DNA in particular single strand breaks (SSB) and alkali-labile sites (ALS). This is calculated by the analysis of the migration of cleaved DNA following contact with an electrophoretic current from the nuclei of the cell. It is this migrating DNA which forms the comet tail (Guillamet *et al.* 2008). The use of Triton-x in the assay lyses the cells, releasing the soluble contents of the cell and eradicating both cellular and nuclear membranes. The bodies become embedded in agarose gel and form what are known as nucleoids due to their resemblance to nuclei. During the process of an electrophoretic field being forced on the cells if a DNA strand break occurs in a loop of DNA then it can stretch in the direction of the anode. Hence if there are further strand breaks then more DNA can migrate towards the anode and so more DNA will be in the comet tail (Azqueta and Collins, 2013). The micronucleus assay however measures genotoxic effects because it is able to expose both clastogenic and aneugenic effects. It is a popular assay due to its simplicity as well as being applicable to a wide range of different cells (Kirsch-Volders, 2011).

Each test has its own advantages and disadvantages and by comparing all of the tests it will be possible to decide on which assay would be the most suitable to use if this was to become a more extensive investigative study. Particularly as this is only a pilot study. As well as this, the study will help to establish whether cells which are exposed to a reduce dosage of chemotherapeutic agents can survive with genotoxic damage.

## **Methods**

Unless otherwise stated all reagents were sourced from Sigma-Aldrich, Dorset, UK.

### **TK6 culture**

TK6 cells were seeded in vented culture flasks at a density of approximately  $3 \times 10^6$  per flask in 10ml TK6 medium (45ml of RPMI, 5ml foetal calf serum, 0.5ml penicillin/streptomycin and 0.5ml L-glutamine). Primary cultures were incubated at 37°C in 5% CO<sub>2</sub>. Cells were then passaged every two days and reseeded at a density of  $3 \times 10^6$ .

### **Chemotherapeutic agent cultures**

At the 7<sup>th</sup> passage, TK6 cells were seeded in a CellStar 12 well suspension culture plate at a concentration of  $5 \times 10^5$  cells per well. The cytotoxicity assays were performed using different concentrations of the agents (for Etoposide; E5-20, E4-10, E3-5, E2-1 and E1-0.1µg/ml and for vincristine; V5-0.5, V4-0.1, V3-0.05, V2-0.01, V1-0.005µM) including the clinically relevant doses (E4 and V4).  $5 \times 10^5$  cells were exposed to 1ml of each of the concentrations of chemotherapeutic agents to a corresponding well with an untreated control set up for each chemotherapeutic agent. The plate was then incubated (37°C/5% CO<sub>2</sub>) for 23 hours.

### **Neutral red assay**

The supernatant was removed from the chemotherapeutic agents' cultures and frozen for later use in the LDH and glucose assays. The tubes were split into 2 so duplicates could be performed. 5µl of triton X was added to the two control tubes (EC and VC). The cells were then washed in 1ml of PBS and 100µl of neutral red medium (5ml of PBS and 50µl of neutral red) was added to each tube. The tubes

were then incubated at 37°C for 2 hours. After the incubation period the neutral red medium was removed from the cells and they were subsequently washed in 150µl of PBS. 150µl of neutral red de-stain solution (10ml ethanol, 9.8ml distilled water and 0.2ml glacial acetic acid) was added to each tube to elute the dye. Each tube was then placed into a 96 well plate along with 2 wells set up for just the de-stain solution. Neutral red absorption was detected at 450nm in a Versa Max micro plate reader.

### **Trypan blue**

The trypan blue assay was performed with different concentrations of the agents as stated previously. 1ml of each of the concentrations was added to a corresponding well with an untreated control set up for each chemotherapeutic agent. The plate was then incubated (37°C/5% CO<sub>2</sub>) for 20 hours.

The different cell concentrations were independently mixed with trypan blue at a ratio of 1:1 and a total cell count was recorded for each drug at each concentration as well as a viable (unstained) cell count using a haemocytometer.

### **LDH assay**

The cytotoxicity induced by etoposide and vincristine was also measured using the LDH assay. 10µl of thawed supernatant (previously frozen from the harvest prior to the trypan blue and neutral red assays) was then added to their own wells for each drug at each concentration in duplicate. To each of the wells containing the supernatant 40µl of 0.75mM sodium pyruvate/β-NADH solution was added but not to the standard wells and the plate was incubated at 37°C for 30 minutes. From here 50µl of 2,4-dinitrophenylhydrazine was added to all wells including the standards and left at room temperature for 20 minutes. From here 50µl of 4M NaOH was added to all wells and a final colour allowed to develop until the absorbance was read at 540nm on a Versa Max micro plate reader. A standard curve was produced from the pyruvate/β-NADH standards.

### **Glucose assay**

Cellular toxicity can be measured through the concentration of glucose in culture media. For example if there is a decrease in the uptake of glucose then this is an indication of a reduction in the cells metabolism (May *et al.*, 2012). A glucose assay was performed to assess glucose consumption by TK6 cells using a glucose oxidase kit adopting a 96 well plate format. Following the harvest of cells exposed to the various doses of etoposide and vincristine before the trypan blue and neutral red assays the supernatant was collected and frozen from each until required for analysis. To a Cell star cell solution 96 well plate, 10µl of sample or standard were added in duplicate as well as 100µl of glucose oxidase/peroxidase reagent. A standard curve was produced by making glucose standards of 0.02, 0.04, 0.08, 0.12, 0.16 and 0.20 mg/ml. 100µl in duplicate was added to two empty wells of the well plate to serve as the reagent blank. To the plate standard or sample, 10µl was added in duplicate followed by 100µl of the assay reagent. The plate was then incubated at 37°C for a total of 30 minutes. Immediately after removing from the incubator the absorbance was read at 450nm using a Versa Max micro plate reader.

### **Alkaline comet assay**

The comet and micronucleus assays were assessed following cultures with the clinically relevant and lowest doses of etoposide (10µM and 0.1µM respectively) and vincristine (0.1µM and 0.005µM).

The comet assay was performed using TK6 cells (approximately 50000 cells in 20-30µl) and re-suspended in 200µl of 0.5% low melting agarose (LMA) and pipetted onto pre-coated microscope slides. The slides were pre-coated in 1% normal melt agarose (NMA). After layering the cells in agarose on the slides a coverslip was placed on top and the agarose was left to solidify at room temperature for 30 minutes. The coverslips were then removed and the slides placed into pre-cooled lysis solution (NaCl, Na<sub>2</sub> EDTA, Tris, 10% DMSO and 2% Triton-X) at room temperature for 1 hour in a foil covered coplin jar to prevent UV damage. The slides were then placed in a horizontal gel electrophoresis tank which was filled with pre-cooled electrophoresis buffer (20mM EDTA, 300mM NaOH, pH 13) for 20 minutes. This allowed the process of DNA unwinding to begin. Following this electrophoresis was performed at 0.7V/cm and 300mA in a cold cupboard for 20 minutes ensuring the slides were protected from light throughout. Once electrophoresis was performed the slides were neutralised twice in Tris at pH 7.5 with 10M HCl. The slides were then stored in a dark cupboard ready to be counted at a later date.

Each slide was stained with 40µl of ethidium bromide (25 µg/ml) immediately before being analysed. The analysis of the slides was performed blind in that the slides had been coded to prevent bias. The slides were viewed under a Texas Red Filter with images collected containing 50-100 cells per treatment. The Perceptive Instruments Comet Assay IV software was used to measure DNA damage.

### **Micronucleus assay**

The trypan blue assay was performed to determine how much culture was needed containing 20 000 cells. This volume for each concentration was re-suspended PBS making the volume up to 150µl. Each drug concentration was pipetted on a pre-polished microscope slide and placed in a cytofunnel. The slides were centrifuged in a Thermoshandon Cytospin 4 (Runcorn, UK) at 1500rpm for 10 minutes. Once completed the slides were allowed to air dry until being fixed in 100% methanol for 8 minutes. The slides were then stored until analysis could be performed.

The following week the slides were analysed. For each slide 20µl of ethidium bromide was placed on the cytopsin and a coverslip placed on top. The slides were analysed using a Leica epifluorescence microscope. 500-1000 cells per slide were analysed blind and a count of micronucleated (MN), apoptotic, blebbed, bi-nucleated and chromosomal cells was taken.

### **Statistical analysis**

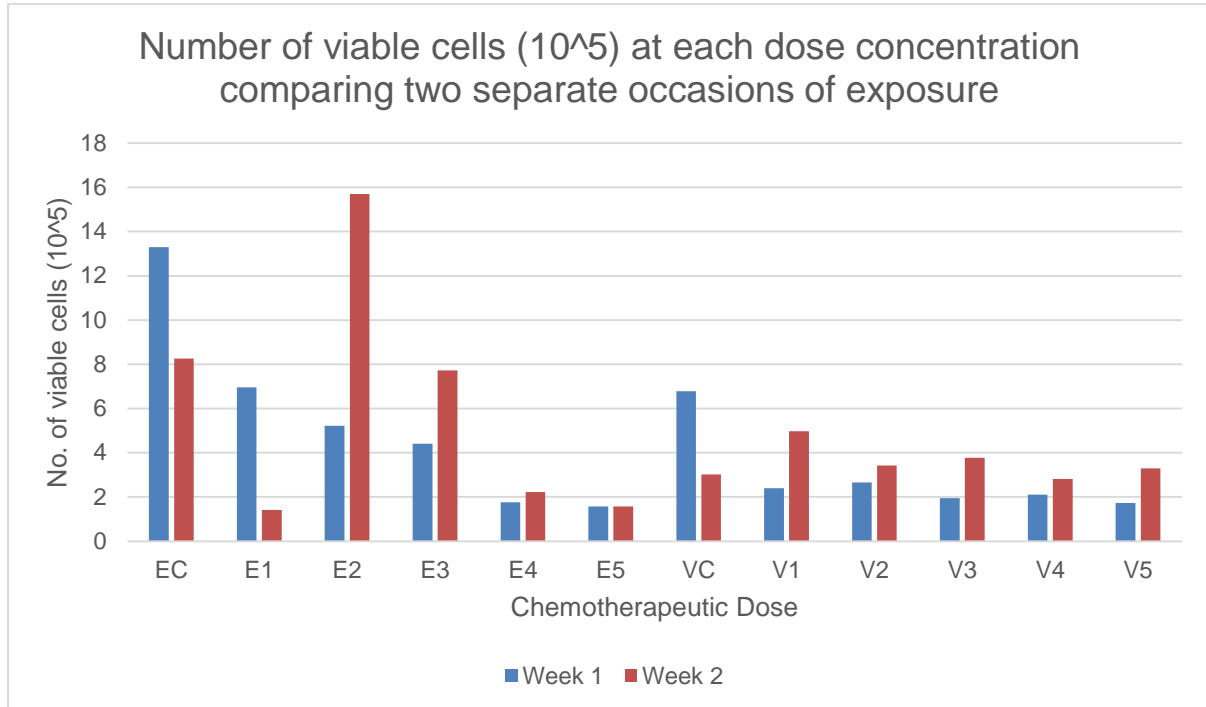
All values cited in the study signify Mean ± SD of the sample unless otherwise stated. All analyses were performed on raw data using SPSS 21. For trypan blue a one sample t-test was performed comparing viable cell numbers at each drug concentration with the control. For all other assays a one way ANOVA was performed followed by a *post hoc* Tukey test with each sample compared with the relevant control, p-Values below 0.05 were considered to be statistically significant. All graphs were produced in Microsoft Excel 2013.



## Results

### Determining cell cytotoxicity

Four different assays were used to measure the cytotoxicity of etoposide and vincristine on the TK6 cells.

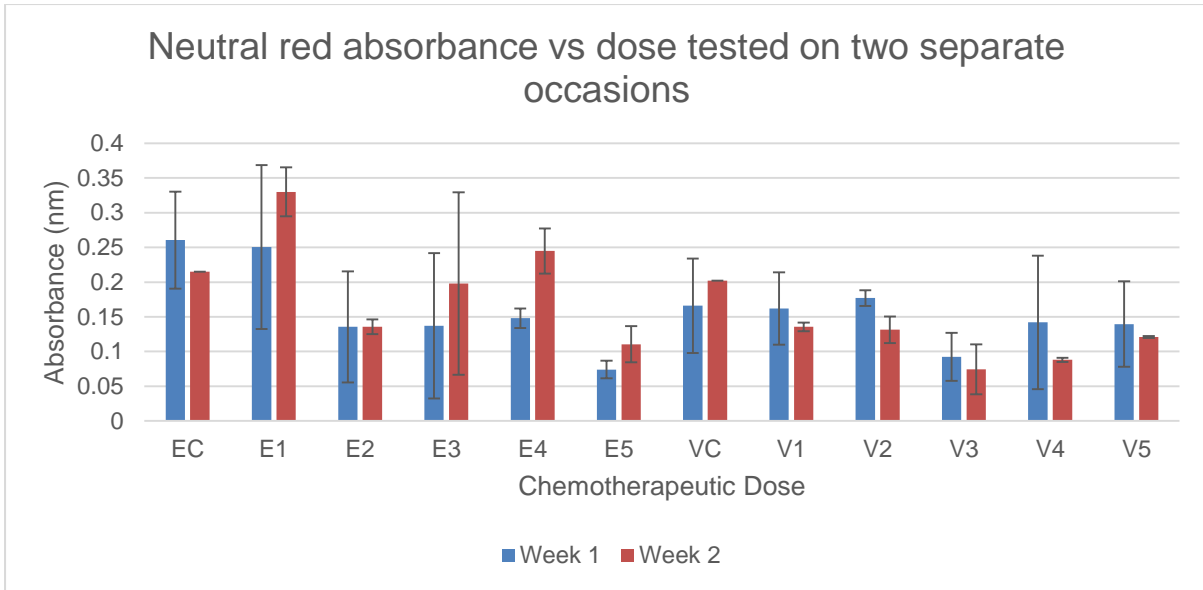


**Figure 1.** Testing cytotoxicity of etoposide and vincristine using the trypan blue assay. Cells were exposed to increasing doses of the chemotherapeutic agents as shown on the graph. The cell counts were performed in duplicate and the number of viable cells at each dose was calculated and plotted on the graph. The graph presents the two repeats for each chemotherapeutic agent.

For the trypan blue assay in the case of the week one repeat there was a greater reduction in the number of viable cell numbers in the case of etoposide but there is also evidence that the untreated control had a considerably higher number of viable cells compared to the vincristine control (Fig. 1). However, due to the fact that there is an extensive range in counts a statistical analysis was unable to be performed to compare the repeats. For E2 a standard deviation  $\pm 7.4$  was produced, such a large SD would discredit the output of such an analysis. However, the week one results can be used to analyse the trend, in general as the chemotherapeutic agents dose increases as well as this a one sample t-test was performed for each of the week one counts in comparison with the relevant control.

### Neutral red

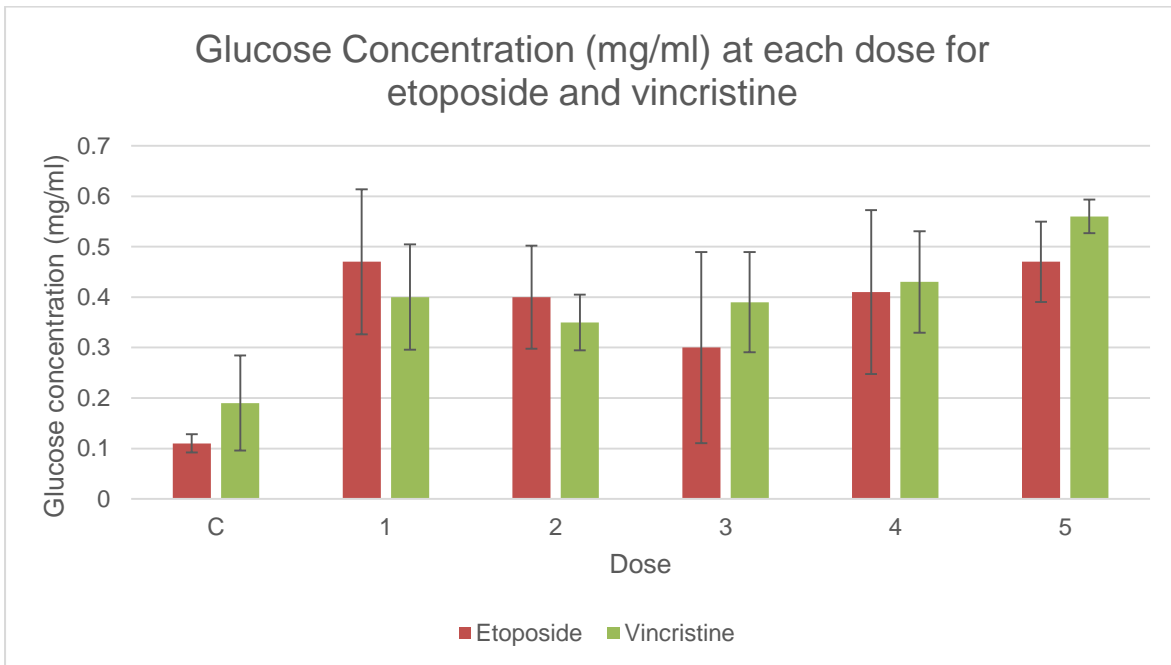
The neutral red assay, however, is an uptake assay so is not looking at the number of viable cells but rather the uptake of the red dye by the cells: the higher the absorbance reading the higher the number of viable cells present. The reading for the control is in fact lower than that of the lowest dose of each of the agents. However, it does fall quite significantly in the case of etoposide more so than for vincristine (Fig. 2).



**Figure 2.** Testing the cytotoxicity of etoposide and vincristine using the uptake assay neutral red.

A one way ANOVA with a *post hoc* Tukey test was performed for both agents. For vincristine there was no significance ( $p > 0.05$ ) but a p-value of 0.033 was obtained indicating significance between the groups. The Tukey test also gave a significant p-value between E1 and E5 (0.029).

**Glucose concentration standard curve**

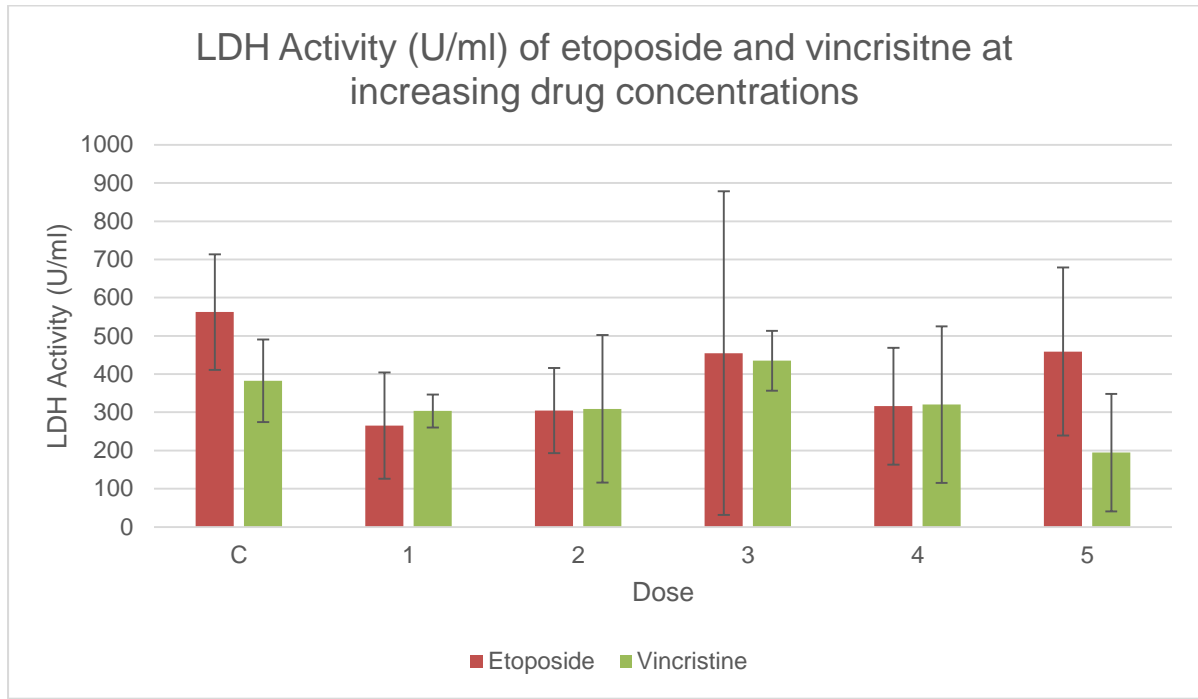


**Figure 3.** Glucose concentration in the supernatant gathered from TK6 cells exposed to increasing doses of etoposide and vincristine Graph represents Mean  $\pm$ SD, n=2.

The glucose concentration graph (Fig. 3) depicts the lowest concentration of glucose in the untreated controls as expected. However, the controls are considerably lower than the first dose particularly in the case of etoposide where there is a sharp rise in

glucose concentration. The highest concentration in both cases is seen in the highest doses of the chemotherapeutic agents. A one way ANOVA with a *post hoc* Tukey test was performed for etoposide and vincristine. Statistically significant p-values were gathered for both agents 0.022 and 0.002 respectively.

**LDH standard curve**

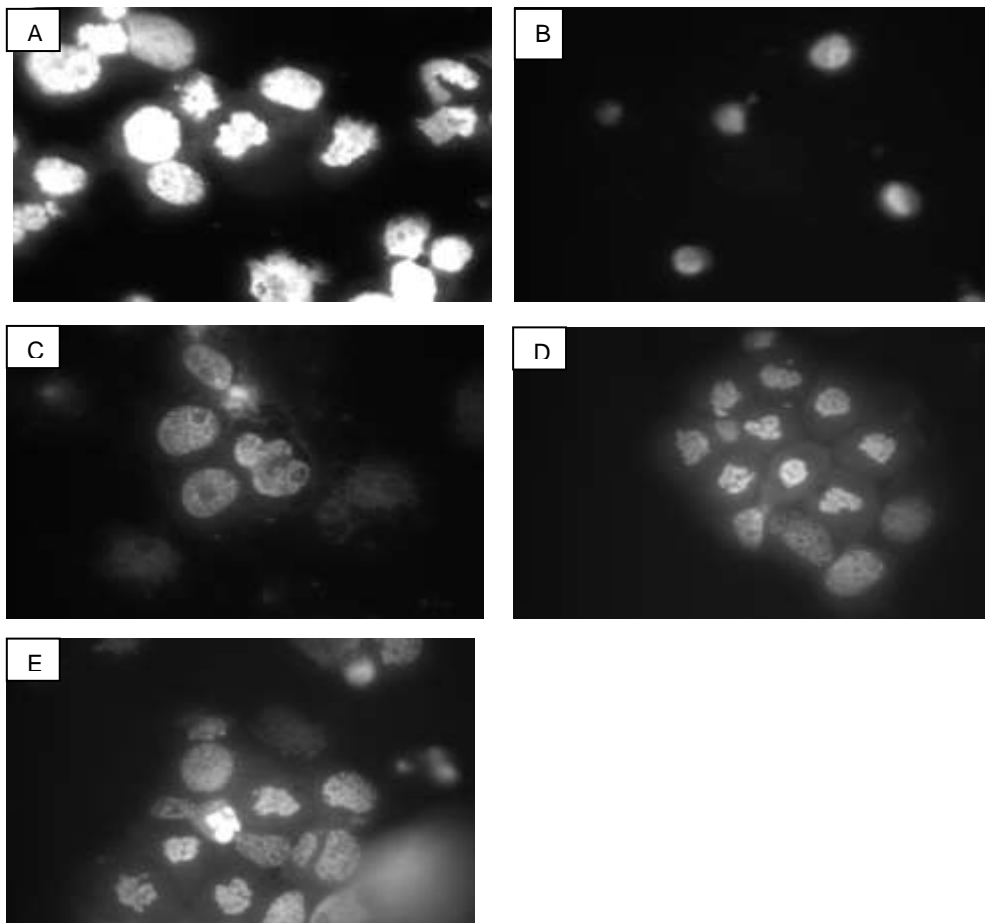


**Figure 4.** Lactate dehydrogenase (LDH) activity determined from TK6 cells exposed to the increasing doses of etoposide and vincristine. Graph represents Mean ±SD, n=2.

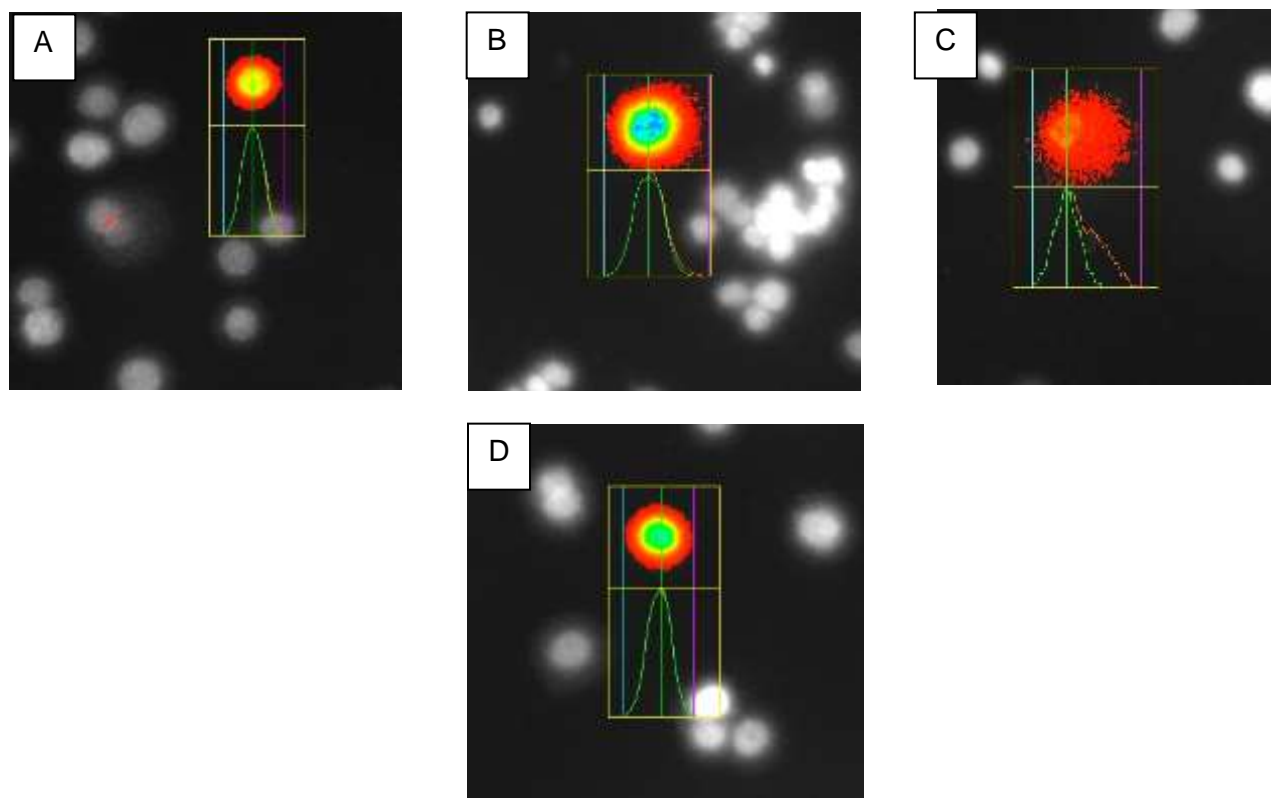
Figure 4, on the other hand, shows the LDH activity of the two different agents plateau along the increasing concentrations more so in the case of etoposide. A one way ANOVA was performed for both chemotherapeutic agents and in both cases there was no statistical significance identified.

**Determining cell genotoxicity using the micronucleus and Comet assays**

The genotoxic effects of etoposide and vincristine can be visualised in the micronucleus assay with a tally of the various characteristics seen at each of the doses; control, lowest dose (E1 and V1) and the clinically relevant dose (E4 and V4) as shown in table 1. When compared with the control each vincristine dose in the case of micronucleated cells has higher values yet apoptotic cells are notably lower than the control in E1, E4 and V1. As well as this V1 displayed a great number of chromosomal cells over a third of the total cells on the slide. Photographs were taken as shown in figure 5 showing the characteristic features highlighted in table 1 alongside images from the control portraying normal cellular features.



**Figure 5.** Morphology of TK6 cells exposed to etoposide and vincristine at low dose and a clinically relevant dose. Normal cell morphology (A) but apoptotic cells can also be seen. (B) shows cells which have been exposed to  $0.1\mu\text{M}$  of etoposide. A cell undergoing apoptosis (C) having been exposed to  $10\mu\text{M}$  of etoposide. An example of a blebbed cell is visible. Cells exposed to  $0.005\mu\text{M}$  of vincristine appeared to have chromosomal aberrations as depicted in (D) there is also a presence of 2 micronuclei and a blebbed cell (F) also shows this and micronuclei can clearly be seen.

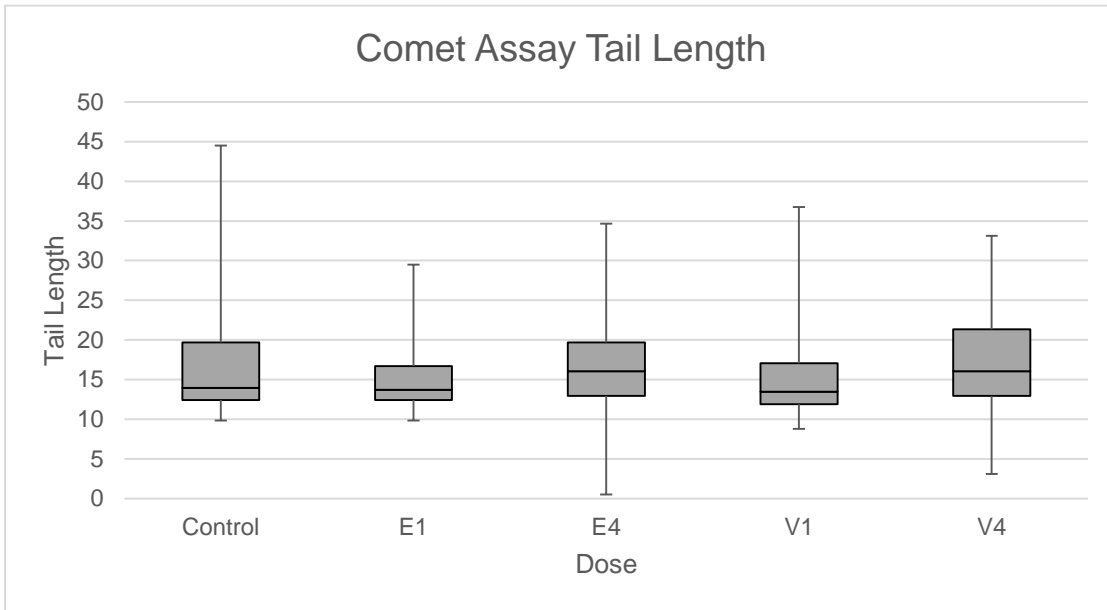


**Figure 6.** Generic images taken from the Alkaline Comet Assay. (A) Control no tail. (B) V4 with moderate tail. (C) E4 with extensive tail. (D) V4 with a moderate tail.

**Table 1.** Quantity of different cell characteristics seen in the micronucleus assay with TK6 cells exposed to an increase in drug concentration. Each sample contained 1000 cells. In both drugs an increase in micronucleated and apoptotic cells is seen when the dose is increased from the lowest to the clinically relevant.

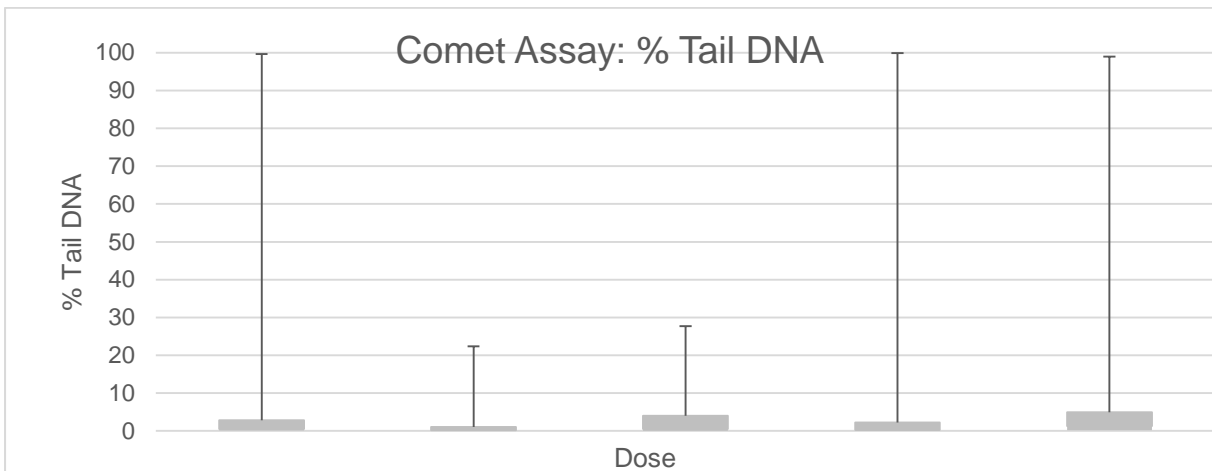
Dose	Micronucleated	Blebbled	Apoptotic	Binucleated	Bridge bleb	Chromosomal
Control	15	25	40	10	5	0
E1	5	5	15	5	0	0
E4	10	10	25	15	0	0
V1	40	4	16	4	0	380
V4	48	0	48	32	0	0

### Comet assay<sup>1</sup>



**Figure 7.** Boxplot of tail length from the alkaline comet assay. Mean tail length appear to be similar for all of the chemotherapeutic agents in comparison with the control. There is a bigger range of length in the comet tail in the control as depicted in the upper quartile. A one way ANOVA was performed etoposide ( $p>0.05$ ) and vincristine ( $p<0.05$ ).

In the case of tail length at both of the higher doses there is an increase in median tail length in comparison to the relevant lower doses and control.



**Figure 8.** DNA damage in the control and the treated samples was assessed by the Comet assay and exemplified by % tail DNA. For etoposide ( $p<0.05$ ) and vincristine ( $p>0.05$ )

<sup>1</sup> Due to sparsely populated cells in the results obtained in the Comet assay previous results from another experiment have been applied.

## Discussion

The assessment of cytotoxicity is important when testing for genotoxicity. Cytotoxicity allows for the maximum concentration of a chemotherapeutic agent to be defined and through genotoxicity testing its capability of causing DNA damage (Shi *et al.*, 2010).

In this study trypan blue was the only cytotoxic assay which could determine the exact number of viable cells as the neutral red is an uptake assay and both LDH and glucose relied upon the assessment of the supernatant. The trypan blue viability assay is based upon the notion that viable cells do not lose their membrane integrity thus do not take up the dye whereas dead cells do stain blue (Strober, 1997). Apoptosis and necrosis are elements which can be responsible for the loss of a cells viability yet during the initial stages of apoptosis a cell may only be seen to possess an irregular membrane (Peter, 2011). For this assay it is expected that as the clinical doses of the chemotherapeutic agents increase, a decrease should be noticed in the number of viable TK6 cells. Figure 1 is a graphical representation of the number of viable cells in comparison to the doses of the chemotherapeutic agents. In the case of etoposide it is visible that there is a high number of viable cells present in the untreated control in that the lowest dose contains almost half the number of viable cells. The viable cell counts for each chemotherapeutic agent confirmed significantly reduced numbers in the presence of high etoposide or high vincristine doses (both  $p < 0.05$ ) from week one data confirming that when exposed to increasing doses of the chemotherapeutic agents cytotoxic effects occur. The results obtained for the second week are unreliable which could be due to a variety of reasons. The trypan blue stain can become toxic to cells over a prolonged period of time which could provide false positive results (Mascotti *et al.*, 2000). In addition to this, trypan blue also doesn't detect apoptotic cells which can give a false negative result with regards to total cell viability (Goktas *et al.*, 2014). Figure 1 also shows that vincristine is more cytotoxic than etoposide due to the decreased number of viable cells compared to the numbers seen in etoposide.

Neutral red is an alternative cytotoxic assay used to assess cell viability. This occurs through the cells uptake of the supravital dye and consequent build up in the lysosomes (Weyermann *et al.*, 2005). The amount of dye which is held is directly proportional to the number of viable cells. The actual uptake of the dye relies upon the cells ability to sustain pH gradients, occurring through ATP synthesis. Therefore, in the case of cell death or if there is a reduction in the pH gradient then the cell is unable to take up the neutral red dye (Repetto *et al.*, 2008). It is considered to have a good sensitivity in the analysis of viability and due to the assay time being less than a few hours is relatively quick in its determination of cytotoxicity (Ciapetti *et al.*, 1996). Yet in comparison to the trypan blue assay the neutral red uptake assay is considerably more time consuming. The assay itself requires few wash steps which contributes to its rapidity but the assay relies upon human efficiency in order to obtain accurate results (van Tonder *et al.*, 2015). The results from this study can reflect this as seen in figure 2, where the absorbance of neutral red at the increasing doses of the agents, etoposide and vincristine varies rather than displaying a decrease in viability as the dose increases. This could be due to a number of reasons such as ensuring the cells were effectively washed in order to remove the neutral red medium (NRM) and the subsequent PBS which could potentially yield false positive results. Furthermore, the effects of the actions of chemotherapeutic

agents which occur are generally intracellular. Thus it is possible that the cells may already be committed to cell death or permanently damaged with an intact cell membrane making it possible for the cells to take up the neutral red dye yet they may actually be non-viable. This could therefore mean that such assays underestimate the damage to the cell again contributing to inaccurate results (Sumantran, 2011).

Cancerous cells possess the ability to metabolically adapt resulting in changes to important cellular processes which can be detrimental in the progression of the disease. These processes can include the need for tightly regulated pH, varying degrees of hypoxia, high LDH levels and low glucose levels, all contribute to the cancer microenvironment and the advancement of the disease (Poder Anderson *et al.*, 2013).

It is widely publicised that the glycolytic rate of cancer cells is high through the increase of glucose uptake. This process is also known as the Warburg effect, first hypothesised by Otto Warburg in 1956 (Zhou *et al.*, 2002). Acute lymphoblastic cells have been demonstrated to upregulate genes such as GLUT1 and GLUT4 which assist in glycolysis and thus contribute to an increased consumption of glucose (Shanmugam *et al.*, 2009). Therefore, glucose has the vital role in the maintenance of the cells homeostasis with regards to its production of energy and also towards its survival thus resisting apoptosis (Shanmugam *et al.*, 2009). However, a study by Zhou *et al.*, (2002) describes that exposing cells to DNA damage inducing agents can cause genes involved in glucose metabolism to be down regulated resulting in the diminished utilisation of glucose. In this same study both etoposide and vincristine were used as DNA damaging agents and in both cases there was evidence of a reduction in both Glut-1 and Glut-3 protein levels.

The purpose of the glucose assay was to determine the concentration of glucose remaining in the medium at each dose allowing the quantification of the metabolic activity of the cells exposed to increasing doses of etoposide and vincristine. Statistical significance was noted for both etoposide ( $p=0.022$ ) and vincristine ( $p=0.002$ ) with a one way ANOVA with a *post hoc* Tukey test. For etoposide there were significant differences between doses compared to the control ( $p<0.05$ ) except in the cases of E2, E3 and E4. There is a vast amount of scatter as illustrated by the error bars in figure 4 (Mean  $\pm$ SD) particularly for E3. In the case of the control, untreated cells, it would be expected that these cells would have a high glucose consumption as they are proliferating and so less glucose in the culture media. Yet in the case of the high doses of etoposide and vincristine as the cells are exposed to considerably higher concentrations of the drug and thus should be undergoing apoptosis as a result, a higher level of glucose should be seen in the media. This is due to the fact that the cells will no longer require the glycolysis products needed to yield ATP as they are no longer proliferating due to their exposure to the cytotoxic agents (Israelsen and Vander Heiden, 2010). However, it is possible to see a decrease in the concentration of glucose left in the media at higher doses due to the fact that the TK6 cells will be actively trying to get rid of the drug and so will metabolise glucose in order to try to eliminate the drug itself. This can be explained through the mechanism of active efflux and drug transporters. Active efflux has become a major component in the disposition of agents and also their activity. To date approximately 20 varieties of drug transporter have been described all of which possess broad specificity for a number of pharmacologically separate compounds. This broad specificity is



accounted for by the fact the substrates are recognised in accordance with their chemical properties (van Bambeke, 2003). In the case of etoposide and vincristine the molecules associated with drug clearance from cells are multidrug resistance-associated protein (MRP) and P-glycoprotein (P-gp). The latter is responsible for the catalysis of the outwards passage of chemotherapeutic agents which is dependent upon ATP (Naito *et al.*, 1999).

One of the most common parameters used in the study of cytotoxicity is the integrity of the cellular membrane. The lactate dehydrogenase assay (LDH) is an example of this as when cells become damaged they release the stable enzyme into the surrounding media (Ivanova and Uhlig, 2008). The amount of enzyme activity is directly proportional to loss of membrane integrity/cell death thus supplying a precise measurement of cytotoxicity by a named agent (Sumantran, 2011). When cells are exposed to conditions which can render them unable to maintain homeostasis, an influx of water and ions occurs causing the cells organelles to swell and lyse. This complete rupture of the cell causes enzymes including lactate dehydrogenase to be released into the extracellular space. The quantification of the activity of such enzymes gives an indication to the amount of necrosis which has occurred (Miret *et al.*, 2006). The LDH release assay has been described as being fast, reliable and is relatively easy to interpret (Fotakis and Timbrell, 2005). However, in this study the LDH assay produced no statistical significance for both etoposide and vincristine ( $p > 0.05$ ) when comparing the individual doses with the control. It is expected that at low concentrations of the chemotherapeutic agents the level of LDH activity in the medium will be low as the majority of the cells will be alive and proliferating. Yet in the higher doses it is anticipated that the agents would cause more damage to the cells which in turn would cause necrosis and damage to the cell membrane thus causing more LDH to escape into the culture media (Iliya and Wallace, 2011). This would also mean the lowest LDH activity should be within the control samples for each drug but figure 4 shows that the LDH activity of all of the chemotherapeutic agent doses are below the control with the exception of V3. A study by Pohjala *et al.*, (2007) noticed that LDH was sub-optimal in determining the cytotoxic effects of agents which did not directly damage cell membranes. The study draws the conclusion that if the agent does not directly damage the cell membrane then it is able to retain its integrity until the latter stages of the cell death pathway. This could be a potential explanation in the plateau seen in figure 4 for the LDH activity of etoposide and in part for vincristine, as these agents are involved in DNA breakage and religation (Jacob *et al.*, 2011) and depolymerising microtubules respectively (Moore and Pinkerton, 2009). Another explanation may be human error particularly in pipetting technique can produce unanticipated results such as lower or higher readings thus adopting an efficient method or using calibrated pipettes and ensuring aliquots are correct should avoid this issue (Sigma-Aldrich, 2013). Not only this but 2,4-dinitrophenylhydrazine is light sensitive which if exposed to UV could impact upon the final result (Miyoshi *et al.*, 2006).

In the case of the genotoxicity assays, the micronucleus assay (MN) is an *in vitro* analysis of aneugenicity and clastogenicity (Kimura, *et al.*, 2013). In this study, MN was used to assess the genotoxic damage of etoposide at its lowest dose (0.1  $\mu\text{M}$ ) and its physiologically relevant dose (10  $\mu\text{M}$ ) and the same for vincristine, lowest (0.005  $\mu\text{M}$ ) and physiologically relevant (0.5  $\mu\text{M}$ ). Considering that the results obtained were only from one experiment and no repeats were carried out statistical analysis was not performed as it was unlikely to be valid. Instead table 1 shows the

number of genetic features observed at the increasing doses in comparison with the control. Etoposide at its lowest dose induced 2/3 less micronuclei than the control and the physiologically relevant dose also didn't exceed the number of micronuclei seen in the control but there was an increase between the two doses. There was also an increase in the number of apoptotic cells compared to the lower dose but again this was lower than the apoptotic cells counted in the control. However, vincristine on the other hand generated over double the number of MN in the lower dose and over three times the amount in comparison to the control at the physiologically relevant dose. Moreover, at the lowest dose of vincristine a considerable number of chromosomal aberrations were seen as shown in figure 6. However, none were recorded in the physiologically relevant dose yet the presence of chromosomal fragments was also noted in a study by Recio *et al.*, (2010). They found that vincristine doesn't induce DNA damage but did cause aneuploidy (chromosomal loss) through abnormal mitoses and micronuclei. Chromosomal material was noticed in the cells treated with vincristine which was likely to be complete chromosomes rather than fragments due to the aneugenic activity of the agent (Witt *et al.*, 2007).

The comet assay is used to assess an agent's capability of inducing genotoxic damage (Shi *et al.*, 2010). In the study the results produced were unreliable in that the cells for each of the chemotherapeutic agent doses were sparsely populated thus could not be used. There are a number of reasons why this may have happened such as the agarose gels coming off of the slides during the comet slide preparation as a result of the normal melt agarose not being heated to the right temperature. A further possibility is the temperature of low melting point agarose was too hot when the cover slips were applied (so not cooled sufficiently to 37°C) causing the cells to overflow to the extremities. However, previously collected results were used from another experiment and tail length and % tail DNA were analysed. % of tail DNA (tail intensity) was analysed as this is the preferred endpoint measure due to its ability to be compared across projects and its relation to DNA damage. In principle if a cell with 0% tail intensity is found this corresponds to no DNA damage likewise a positive increase in percentage indicates further DNA damage. Cells with a percentage greater than 85 represent dead or dying cells (Bright *et al.*, 2011). From figure 7 it is visible that the control and both doses of vincristine have a range >85% thus indicating the presence of dead/dying cells. However the high proportion of DNA damage in the control could be a result of already necrotic cells thus already having DNA damage being taken into the assay. Prior to performing the Comet assay a cell count was performed using trypan blue on a 20µl aliquot of each dose. At each concentration a number of non-viable cells were recorded in the aliquot meaning already necrotic cells would be taken into the comet assay before DNA damage was assessed. This could also account for the increased tail length of the control in figure 6. Etoposide constructs a ternary complex with DNA through the inhibition of topoisomerase II activity causing single and double strand breaks as it prevents the enzyme from religating (Wu *et al.*, 2011). A study by Godard *et al* (1999) showed that etoposide caused DNA fragmentation but that it was possible that these cells were not in fact dead cells but that they did have stable ternary complexes. Moreover, a potential drawback of the assay itself is that it may produce bias results through cells being able to repair DNA damage in various phases of the cell cycle (Kruszewski *et al.*, 2012). On the other hand, the comet assay is regarded as an adaptable and highly sensitive method to assess the genotoxic damage occurring in

cells and has been adapted over the last two decades to evaluate a range of DNA damage including both single and double strand breaks (Kruszewski *et al.*, 2012).

From carrying out these assays their relevance in the current field is noticeable particularly in the case of reduced intensity conditioning. This study has shown that lower doses of chemotherapeutic agents although cytotoxic can survive with genotoxic damage as seen with the lowest and clinically relevant dose of etoposide.

## **Conclusion**

Overall, trypan blue and the Comet assay are the best cytotoxic and genotoxic assays if this was to become an investigative study. This is due to the rapidity and effectiveness including cost of the trypan blue assay to detect cytotoxicity by quantification of viable cells. As well as this, the comet assay has shown that vincristine is a more cytotoxic agent than etoposide but dose dependent increase in DNA damage was visible in the latter. Not only this but the comet assay can easily be performed subsequently after a trypan blue viability assay allowing the characterisation of cytotoxicity and genotoxicity of named agents to be prompt and effective. However, this was only a pilot study due to a lack of repeats, statistics and chemotherapeutic agents. Subsequent analyses would need to be undertaken to confirm the results obtained in this study.

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