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## Analysis of the repair of topoisomerase II DNA damage

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Analysis of the Repair of Topoisomerase II DNA Damage

by

ERIC D. GOLDSTEIN

A thesis submitted in partial fulfillment of the requirements  
for the Honors in the Major Program in Microbiology and Molecular Biology  
College of Medicine  
and in The Burnett Honors College  
at the University of Central Florida  
Orlando, Florida

Spring Term 2011

Thesis Chair: Dr. Mark T. Muller

## Abstract

A large number of anti-cancer chemotherapeutics target DNA topoisomerases. Etoposide is a specific topoisomerase II poison which causes reversible double strand DNA breaks. The focus of this project is to analyze the repair of DNA damage induced by etoposide.. Double strand DNA break repair is mediated by through either non-homologous end joining (NHEJ) or homologous recombination. NHEJ repairs through direct ligation of a double stranded break while homologous recombination utilizes a homologous template to recover the wild type sequence. A reporter cassette, RYDR-GFP, has been stably integrated into HeLa cells. This reporter contains an ultra-high affinity topoisomerase II cleavage site (RY) placed in the middle of a mutant GFP sequence. Flanking this sequence is a corresponding stretch of wild type GFP that is used as template to repair the break and restore gene function yielding GFP positive cells. Titrations with etoposide have shown that a logarithmic increase in drug concentration yields a corresponding increase in repair through homologous recombination (HR). This result demonstrates that topoisomerase II mediated damage is efficiently repaired by the process of HR. To examine NHEJ repair, a doxycycline inducible, stably integrated NHEJ HeLa cell reporter cassette was also evaluated. The data indicates that repair of topoisomerase II mediated DNA damage occurs more efficiently through the HR pathway. Collectively, the data suggests that tumor cells proficient in HR repair may effectively elude treatment by topoisomerase II targeting drugs.

## **Acknowledgements**

I would like to especially thank Dr. Mark T. Muller for the constant guidance not only for this project, but for my future and life in general. Dr. Muller has been a constant inspiration and a role model throughout. I would also like to thank Dr. Ken Teter for constant revisions, support, and practice in data presentation; all of which are invaluable. Dr. Jack Ballantyne has also been graciously supportive throughout this process and taking time out of his schedule to be a part of the thesis committee.

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## Chapter One: Introduction

Topoisomerase poisons are widely used as anti-cancer chemotherapeutics [2].

Topoisomerase II $\alpha$  (topoII $\alpha$ ) is the target for many anti-cancer agents because cancer cells have increased mitotic activity, requiring an increase in topoII $\alpha$  expression.

### *Topoisomerase II $\alpha$ Mechanism*

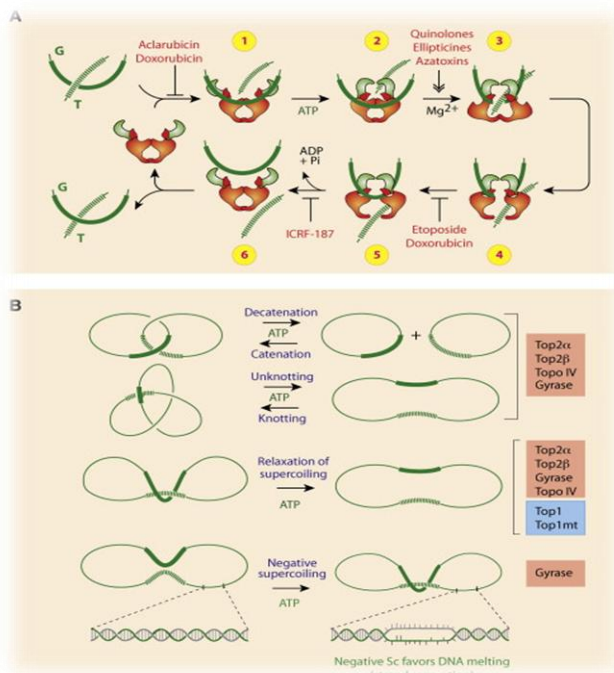
The role of topoII $\alpha$  is in relaxation, decatanation, and unwinding DNA during replication and cellular division (**Figure 1**). A prime example of topoII $\alpha$  catalytic activity is during DNA replication when the replication fork melts the DNA hydrogen bonds between base pairs. As a result, the DNA preceding the replication fork begins to wind into a highly taut coil called a supercoil. If left alone, this negative supercoiling can be so severe as to fracture the DNA itself, thereby creating a genotoxic event [2]. TopoII $\alpha$  relaxes the supercoiling.

TopoII $\alpha$  is homodimer with a Mg<sup>2+</sup> cation in each dimer. TopoII $\alpha$  conformationally exists as closed or open clamps dependent upon ATP binding. ATP binding switches open to closed clamp formation. In each subunit, the Mg<sup>2+</sup> cation stabilizes, in humans, the tyrosine – 804 residue in allowing a nucleophilic attack of the 5' phosphodiester bond [2]. The mechanism is repeated on both sides of the double helix. TopoII $\alpha$  is now covalently bound creating a protein-DNA adduct with a double stranded break (DSB). Transient strand passage translocates the uncut strand through the DSB. Within the active site, the dissociated ends are religated. ATP hydrolysis then switches the homodimer to the open conformation. This mechanism is equilibrated and can either increase or decrease the linking number by two. The isozyme topoII $\beta$  is not mitotically stimulated and is poorly understood, however shares this mechanism [2] (**Figure 1**).

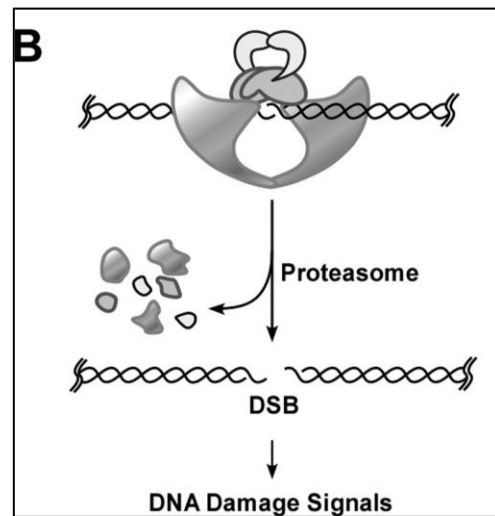


### Topoisomerase I Mechanism

Topoisomerase I (topoI) has importance in mitotic, transcription, and promoter regulation [2]. TopoI does not require ATPase activity. A tyrosine residue performs a nucleophilic attack on the 5' phosphodiester bond, creating a single stranded gap. TopoI transfers the free 3' end about the intact strand and religates the gap within the catalytic site. The topoI mechanism is in equilibrium, allowing for the increase or decrease in linking number by one.



**Figure 1 - A. Topoisomerase II Poison; B. Topoisomerase II Enzyme Mechanism [2]**



**Figure 2 - TopoII Cleavage Complex Repair [4].**

### *Topoisomerase II $\alpha$ Poisons*

TopoII poisons such as etoposide (VP16) stabilize enzyme/DNA cleavages and fragment the genome (**Figure 1**). Many topoII $\alpha$  agents are in clinical use and are FDA approved. Therefore, it is vital that we understand how topoII $\alpha$  breaks are repaired. VP16 itself is a widely used chemotherapeutic agent [5].

Chemotherapy sometimes requires high dosages of topoII $\alpha$  agent in order to ensure that DNA damage does not undergo repair, as the cleavage complex is a transient and reversible event [2]. The stabilized DSB created by topoII $\alpha$  poisons increase the half life of the cleavage complex. DNA/topoII complexes are processed by the 26S proteasome, a macromolecular structure that degrades ubiquitinated proteins, thus removing the topoII $\alpha$  polypeptide portion and leaving a DSB (**Figure 2**) [4]. Recent studies further indicate that the removal of the topoII $\alpha$  protein can be performed through CtIP and the phosphodiesterases TDP1 and TDP2 [9]. If the DNA damage is not efficiently repaired, the cell will undergo apoptosis. This could possibly reduce the amount of agent needed to fight the malignancy. Information on the repair process can lead to new strategies that can inhibit the reversal of topoII $\alpha$  mediated DNA damage, thereby minimizing patient side effects through the increase of drug efficacy.

### *DSB Repair Pathways*

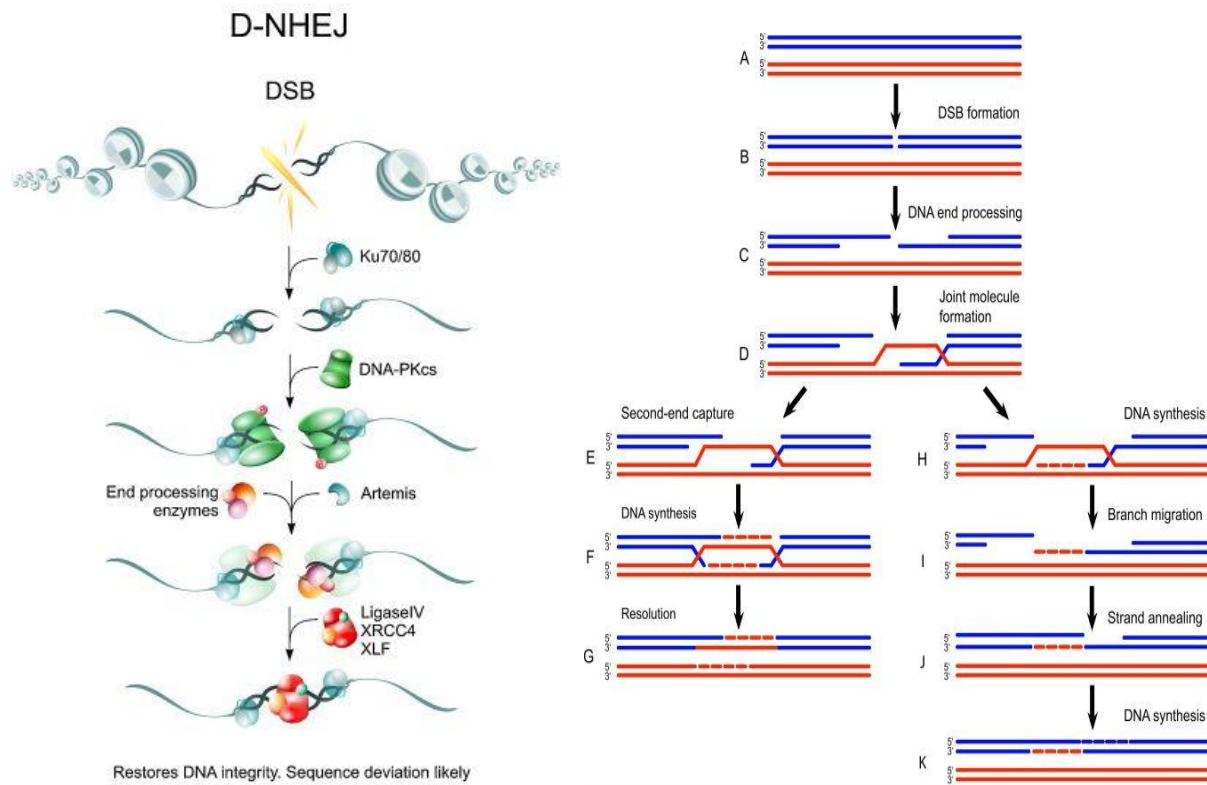
DSBs are common events. The dissociated ends can reassociate indiscriminately, differing in sequence from the wild type. The DSB ultimately can lead to chromosomal translocations [7]. To circumvent this, cells evolved two known mechanisms to correct the DSB, non-homologous end joining (NHEJ) and homologous recombination (HR).

NHEJ is the main pathway by which healthy cells repair from DSBs; however this can alter gene regulation or expression (**Figure 3**). The process involves the direct ligation of a DSB without regard to sequence homology or cell cycle [12]. NHEJ is a low fidelity, high mutation prone pathway, but repairs DSBs rapidly [12]. Ku, a heterodimer of Ku70 and Ku80, recognizes the DSB and initiates the NHEJ repair pathway [12]. The Ku protein attracts DNA-PKcs forming a holoenzyme and autophosphorylates itself, possibly providing the energy needed for the subsequent blunt ligation. NHEJ provides genomic stability with a half life of 30 minutes [12].

HR is a high fidelity pathway which uses a template donor sequence to reverse a DSB. HR commences upon DNA damage recognition and a cascade of signaling recruits proteins that further resect the break to single stranded 3' ends (**Figure 3**) [8]. The single stranded ends are then coated with single stranded binding proteins, protecting the templates. These unbound ends are then wrapped with Rad51, which is associated with BRCA1. With Rad51 bound, the single stranded DNA participates in homology recognition [1]. The Rad51 complex also allows for strand exchange. Subsequent branch migration and nucleotide polymerization from DNA polymerase II occur. The whole complex then resolves itself with an exact copy of the template homolog where the DSB occurred.

### *Goal*

The goal of this work is to analyze if topoII/DNA damage complexes are repaired through either HR or NHEJ. HR and NHEJ events resulting from poison damage can be quantified through the use of a highly specific reporter cassette.



**Figure 3 - NHEJ [11] and HR [7] Pathways.** NHEJ uses the Ku complex to recruit subsequent proteins such as DNA-PKcs, ultimately resulting in blunt double strand ligation of the DSB. HR is a high fidelity pathway that uses a donor sequence as a template, thus resulting in high fidelity DNA retrieval.

## Chapter Two: Methods and Materials

### *Exposure of DR-GFP HeLa cells to Etoposide and ISce-I*

Stably integrated DR-GFP (HR) HeLa cells were maintained in 1640 RPMI (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) in a humidified 37°C with 5% CO<sub>2</sub> incubator. Cells were dispensed into three 12 well plate formats at 200,000 cells per well in 2 mL 1640 RPMI 10% FBS. Following 24 hours incubation, six of the twelve wells were transfected with 1.2 µg pISce-I and 3.6 µL Lipofectamine Reagent 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions for four hours in 1 mL of OPTI-MEM I (Invitrogen). Next, the OPTI-MEM I was aspirated from the transfected wells and dispensed 2 mL 1640 RPMI 10% FBS, then incubated for 24 hours. After incubation, the 1640 RPMI from all twelve wells were aspirated and 1 mL OPTI-MEM I was added to each well with the desired concentration of VP16 from a 20 mM aliquot stock solution (TopoGEN). The cells were treated with VP16 for one hour, then washed with 1 mL 1X PBS, and 2 mL of 1640 RPMI 10% FBS was added. The 12 well plates were incubated for 24 and 72 hours to establish a time course for recovery. At the end of each respective recovery incubation time, 200 µL of 0.25% Trypsin-EDTA (Invitrogen) was added and incubated for 5 minutes. Next, 200 µL of 1640 RPMI without FBS was added to neutralize the trypsin and 400 µL of the cell suspension was dispensed in 400 µL 1X PBS in a 5 mL Fluorescence Activated Cell Sorter (FACS) tube (FALCON). GFP was read using FACSCalibur and CellQuest software (BD Bioscience, San Jose, CA).

### *Screening of RYDR-GFP clones*

HeLa cells were stably integrated with RY-DRGFP (RY-HR) plasmid (TopoGEN) by Alex Fagenson, Dr. Muller laboratory. Twenty-four hours prior to transfection, the HeLa cells were plated at a concentration of  $5 \times 10^5$  cells per well in a six well plate. The HeLa cells were transfected in six-well plates with Lipofectamine 2000 reagent according to manufacturer's instructions. Next, two  $\mu\text{g}$  of plasmid DNA, RY-DRGFP #1 and #6 respectively were transfected in 1 mL OPTI-MEM I. Four hours following transfection, the media was aspirated and cells were grown in 1640 RPMI 10% FBS for twenty four hours. Cells were then washed with 1X PBS, trypsinized, and split into 10 cm culture dishes. Clones were subjected to single colony isolation technique while being maintained in media containing puromycin ( $2 \mu\text{g}/\text{mL}$ ) and grown until the colonies were visible on the plate (~4 weeks). The 1640 RPMI 10% FBS media was changed routinely for both plasmid cultures every four days. Colonies were then picked with a pipette tip and placed in 48-well tissue cultured plates. Cells were incubated and forty eight clones of plasmid #1 and #6 were maintained in 48 well formats until clone screening was performed.

Colonies were selected as single clones and were subcultured and grown up to expand the clones. Healthy cells were plated in six, 12 well plates. One colony was placed per well in two 12 well plates. Approximately 200,000 cells per well were then plated and incubated for 24 hours with puromycin containing 1640 RPMI. Following this,  $1 \mu\text{g}$  pISce-I with Lipofectamine Reagent 2000 was transfected into one of the 12 well plates according to manufacturer's instructions in 1 mL of OPTI-MEM I for five hours. The media was then aspirated and replaced with 2 mL 1640 RPMI 10% FBS and incubated for 24, 72, and 144 hours. Using the FACSCalibur and CellQuest software, GFP expression was calculated. The best clone was kept for further projects, and the remaining clones were discarded.

*Confocal Microscopy.*

Two 6 well plates were seeded with approximately 100,000 cells per well on top of cover slips in four of the six wells and 2 mL of 1640 RPMI 10% FBS media. The plates were then incubated for 24 hours at 37°C, 5% CO<sub>2</sub>, in a humidified incubator. The media was then aspirated in wells one, three, and four and replaced with 1 mL OPTI-MEM I. To well one, 2 µg ISce-I and Lipofectamine Reagent 2000 for the non-inducible RY clone was added according to manufacturer's instructions for four hours, and 2 µL of doxycycline for the inducible system was incubated until harvesting. To well three and four, 5 µM and 100 µM of VP16 respectfully was exposed to the cells for one hour. Following drug treatment, the media was aspirated and replaced with 1640 RPMI 10% FBS and incubated for 24 and 48 hours to establish a time course. After the desired incubation time, the media was aspirated and the cells were washed with 1X PBS. Using a bent hypodermic needle, the cover slips were carefully removed. A 1.5 µM PI staining solution stained the cells for thirty minutes in the dark. The cover slips were then washed with 1X PBS. Using FischerFinest Premium Slides, one drop of emulsion oil was placed between the slide and the cover slip. A KimWipe was used to remove excess oil from around the cover slip. Four drops of clear nail polish was dispensed and a ring was made around, not overlapping with, the cover slip and allowed to dry for five minutes. Then two drops of clear nail polish were placed on the edge of the cover slip and using the supplied brush, the nail polish was gently made to overlap onto the cover slip. The slides were dried for five minutes. The slides were then analyzed using a confocal microscope at 20X and 100X power.

#### *Analysis of GFP and Methylation in RY-HR Stably Integrated HeLa Cells Using VP16*

Approximately  $1.37 \times 10^6$  RY-HR HeLa cells per 100mm plate were incubated in 10 mL of 1640 RPMI 10% FBS for a total of 9 plates. The colonies were incubated at 37°C, 5% CO<sub>2</sub>, and in a humidified incubator environment for two days. Making sure the cells were in heavy exponential growth, 0.5 μM and 5 μM VP16 were added to two of the plates and exposed continuously for five days until confluent. In four plates, 0.5 μM, 5 μM, 50 μM, and 500 μM of VP16 were exposed for one hour in 2 mL OPTI-MEM I media, then aspirated, and replaced with 10 mL 1640 RPMI 10% FBS media and incubated for five days. One plate was left untreated, and the last plate was exposed to 5 μg of ISce-I for 4 hours with Lipofectamine Reagent 2000 according to the manufacturer's instructions in 5 mL OPTI-MEM I. The media was then aspirated and replaced with 1640 RPMI 10% FBS media and incubated for five days until confluent. Using the FACSCalibur and CellQuest software GFP expression was read.

#### *Trypan Blue Exclusion Assays for Cell Viability*

RY-HR HeLa cells and NHEJ reporter HeLa cells were plated at approximately 500,000 cells per well and incubated for 2 days followed by treatment with increasing VP16 concentrations. Typically, the cells were exposed to VP16 for one hour. The cells were then washed and incubated in 1640 RPMI 10% FBS medium for three days. After the three day incubation, 180 μL of 0.1% trypan blue dye was aliquot to microcentrifuge tube. The supernatant of each well was saved in a 15 mL conical tube. The cells were trypsinized with 200 μL of 0.25% Trypsin-EDTA (Invitrogen) and incubated for five minutes under humidified, 37°C, 5% CO<sub>2</sub> incubator conditions. To neutralize the trypsin, 200 μL of 1640 RPMI was added to the trypsinized cells creating a pool of cells, which was then added to the supernatant. From the cell



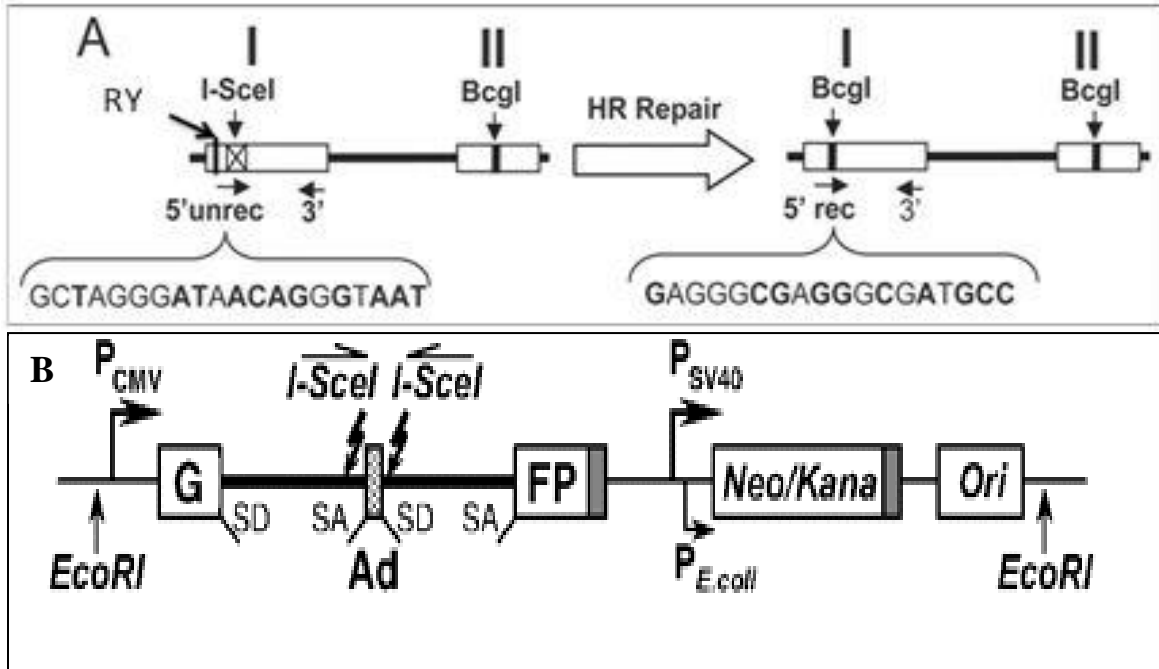
suspension, 20  $\mu\text{L}$  was added to the 180  $\mu\text{L}$  of 0.1% trypan blue dye. From that, 10  $\mu\text{L}$  of the dyed cells was injected into a hemocytometer slide and viewed under a microscope. The slide is divided into four quadrants and each of the four quadrants was counted and totaled. A ratio of stained versus unstained cells indicates percentage cytotoxicity.

#### *Analysis of Repair of Topoisomerase I and II DNA Damage by HR and NHEJ Pathways*

Approximately  $1 \times 10^6$  cells of both stably integrated RY-HR HeLa cells and inducible, stably transfected NHEJ reporter cassette HeLa cells were plated in 11 plates for the RY-HR HeLa line and 10 plates for the NHEJ HeLa line. The cells were incubated for three days in 5%  $\text{CO}_2$ ,  $37^\circ\text{C}$ , and humidified conditions. Following incubation, the cells were observed to be in exponential growth. The plates were exposed to an increase in VP16 concentration, and an increase in camptothecin (CPT), a topoisomerase I poison for one hour in 2 mL of OPTI-MEM I. A positive control and a transfection efficiency control were set up by the transfection of pISce-I and pEGFP utilizing Lipofectamine 2000 reagent, according to manufacturer's instructions. One plate for both lines was left untreated and untransfected as a negative control. The plates were then incubated for 3 days following the drug treatments. After incubation, the supernatant of the plates was transferred to a 15 mL conical tube. The cells were then trypsinized with 1 mL 0.25% Trypsin-EDTA (Invitrogen) for 5 minutes in  $37^\circ\text{C}$ , 5%  $\text{CO}_2$ , and humidified incubator conditions. The trypsin was then neutralized with 1 mL of 1640 RPMI without FBS, and 1 mL of the trypsinized cells was added to 1 mL 1X PBS in a 5 mL FACS tube (FALCON). The cells were analyzed via FACSCalibur and CellQuest Pro (BD Software) for GFP expression.

*Doxycycline inducible ISce-I expression system*

The inducible reporter system used for the NHEJ and HR HeLa cells lines were stably integrated by Dr. Bongyong Lee. These reporters use a tetracycline induction system to transcribe ISce-I endonuclease. Doxycycline is a semi-synthetic tetracycline derivative. Following exposure to doxycycline, the pCMV promoter sequence is exposed allowing for transcription of the ISce-I downstream gene. Without doxycycline exposure, no ISce-I is expressed.



**Figure 4 – The RYDR-GFP and NHEJ Reporter Constructs.** (A): RYDR-GFP reporter system. The RY element and the ISce-I loci create DSBs when exposed to a topoII $\alpha$  poison or ISce-I restriction endonuclease respectively. The First cassette following a DSB searches for homology amongst the second cassette, leading to HR repair of the DSB and GFP expression. Before HR repair, cassettes 1 and II are incapable of expressing GFP due to mutations in the first cassette and a stop codon in the second cassette. Following HR repair as dictated by the diagram, cassette I can express GFP. (B): The NHEJ reporter system. The opposing ISce-I restriction cut sites allow for direct ligation of the opposing cassettes initiating GFP expression. The RY sequence is not needed due to the size of the NHEJ reporter system.

## Chapter Three: Results

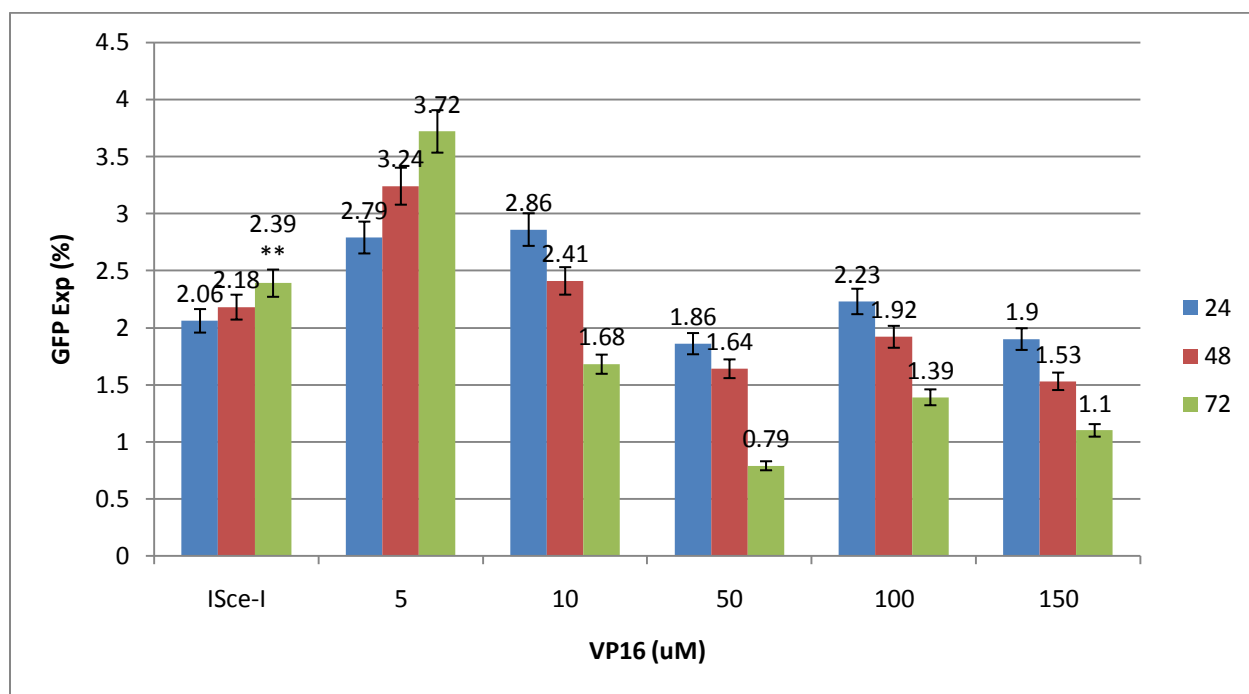
### *HR analysis following VP16 drug treatments and transfection of ISce-I in HR HeLa cells*

The treatment of stably integrated HR HeLa cells with VP16 served as an initial and essential founding piece of information for the following results (**Figure 5**). The positive control serves to illustrate the reporter system is working. The positive control, which includes the transfection of ISce-I with no subsequent drug treatment, shows the potential of GFP expression. The negative control consists of HR cells, lacking ISce-I transfection and drug treatment, serving as a background reading and is ultimately subtracted from the corresponding ISce-I data point. Although the anticipated GFP expression for the negative control should be 0%, the 0.1% GFP finding is likely due to the nature of the integrated GFP cassettes.

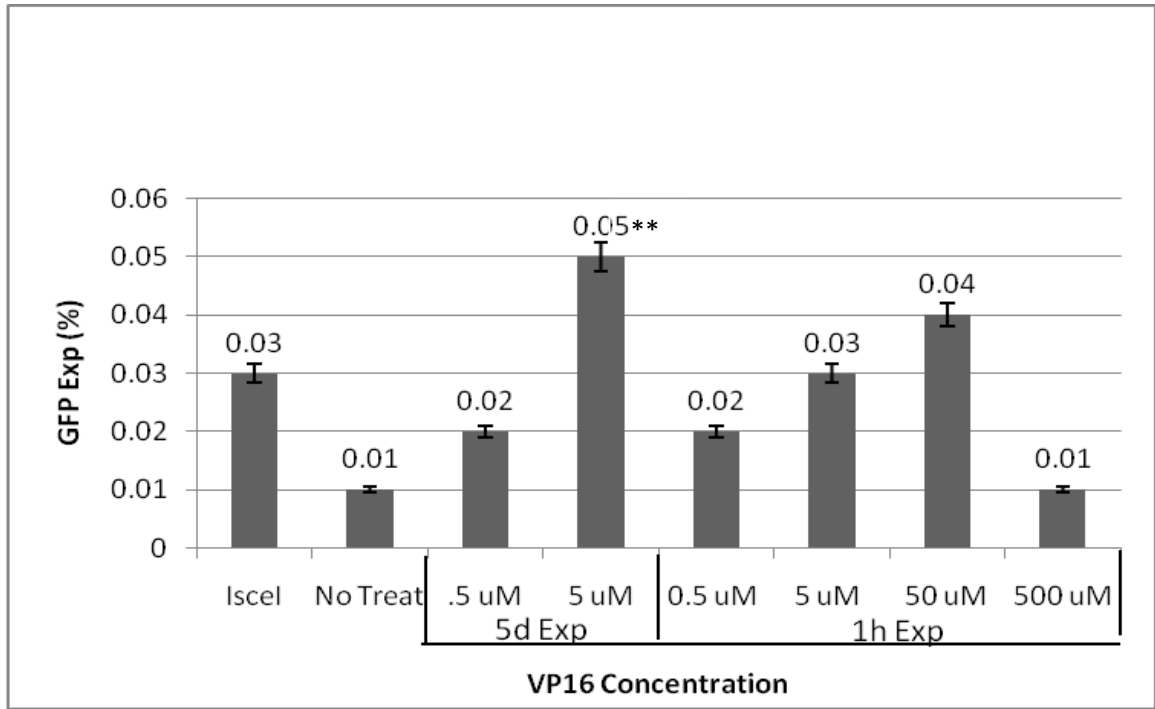
The combination of VP16 with the transfection of ISce-I increased the GFP expression by 1.3% in the 5  $\mu\text{M}$  etoposide range, and steadily declined with higher VP16 concentrations thereafter (**Figure 5**). In addition, GFP expression gradually decreased over time with VP16 concentrations above 5  $\mu\text{M}$ . However, the remaining increasing VP16 and ISce-I exposures were gradually decreased through both time and increasing VP16 concentration. This is likely due to toxicity of the VP16 drug ( $\text{IC}_{50} = 200 \mu\text{M}$ ).

Following this data, a plasmid that narrows variability was used. The RYDR-GFP (RY-HR) (**Figure 4**) cassette contains an ultra high affinity 56 base pair purine rich sequence for topoII $\alpha$  [10]. This high affinity topoII binding site should attract the endogenous topoII and direct cleavages in this region 5' of the ISce-I site in the presence of a drug such as VP16. As a result, the cellular DNA damage repair system should be activated with the HR cassette. The results show that HeLa cells are able to repair the VP16 initiated DSB up to 5  $\mu\text{M}$  concentration

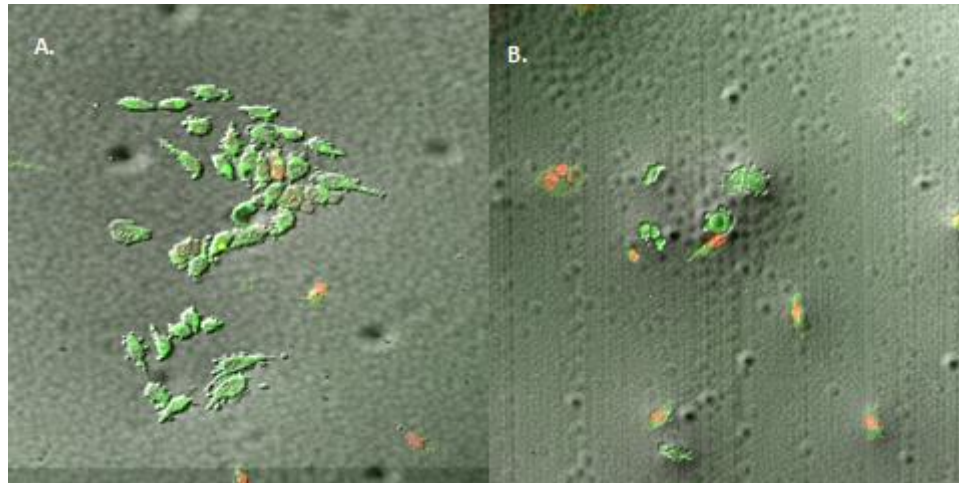
**(Figure 6).** Two exposure times of VP16 were allotted for the stably integrated RY-HR HeLa cells. One batch of cells was exposed to VP16 for 5 days. The second batch was exposed to VP16 for 1 hour and then allowed to recover. The positive control is solely a transfection of ISce-I plasmid while the negative controls represent GFP expressed in the absence of ISce-I and drug treatment. There is a clear trend with increasing concentration of VP16 treatment in both the prolonged and short drug exposure time frames **(Figure 6)**. During the five day exposure, there is an increase of GFP beyond the positive control. The one hour drug treatments also had an increasing trend with increased dosage of VP16 **(Figure 6)**. The data suggest that the presence of the RY element is stimulating HR in the GFP reporter through VP16 drug treatment. In this analysis, cells were treated at low to high levels of VP16 for a total of 5 days or with the same concentration for 1 hour followed by 5 days of recovery. In both cases, it is clear that VP16 is inducing the formation of WT GFP<sup>+</sup> cells at certain concentrations (0.05 – 50  $\mu$ M). Higher concentrations were toxic to the cells. As a result of this, we decided that a morphological investigation of the GFP expressed in HR, RY-HR, and NHEJ HeLa cells was required.



**Figure 5 - HR in VP16 Treated HR HeLa Cells.** Stably integrated HR HeLa cells were transfected with ISce-I and treated with VP16 for 1 hour as described in the “Materials and Methods.” In a twelve well format, approximately 200,000 cells were seeded per well and incubated for 24 hours. Transfection of ISce-I for 4 hours occurred in the necessary wells for four hours. The cells were then incubated for 24 hours and a 1 hour VP16 drug treatment ensued. A time course for recovery for 72 hours (\*\*p<0.01) was read for GFP expression using FACS analysis. Error bars symbolize data range after three replicated trials.



**Figure 6 - HR in VP16 Treated RY-HR HeLa Cells.** Stably integrated RY-HR HeLa cells were seeded at approximately  $1.37 \times 10^6$  cells per plate and incubated for 24 hours. Following incubation, drug treatments lasting for the indicated time (5d = 5 days, 1h = 1 hour) at the stated VP16 concentrations. One hour drug treated cells were washed with 1X PBS, then incubated for 5 days. The positive control was transfected with ISce-I for four hours, and recovered for 5 days. All cells were harvested simultaneously for GFP analysis via FACS (\*\* $p < 0.4$ ). Error bars symbolize data range after three replicated trials.

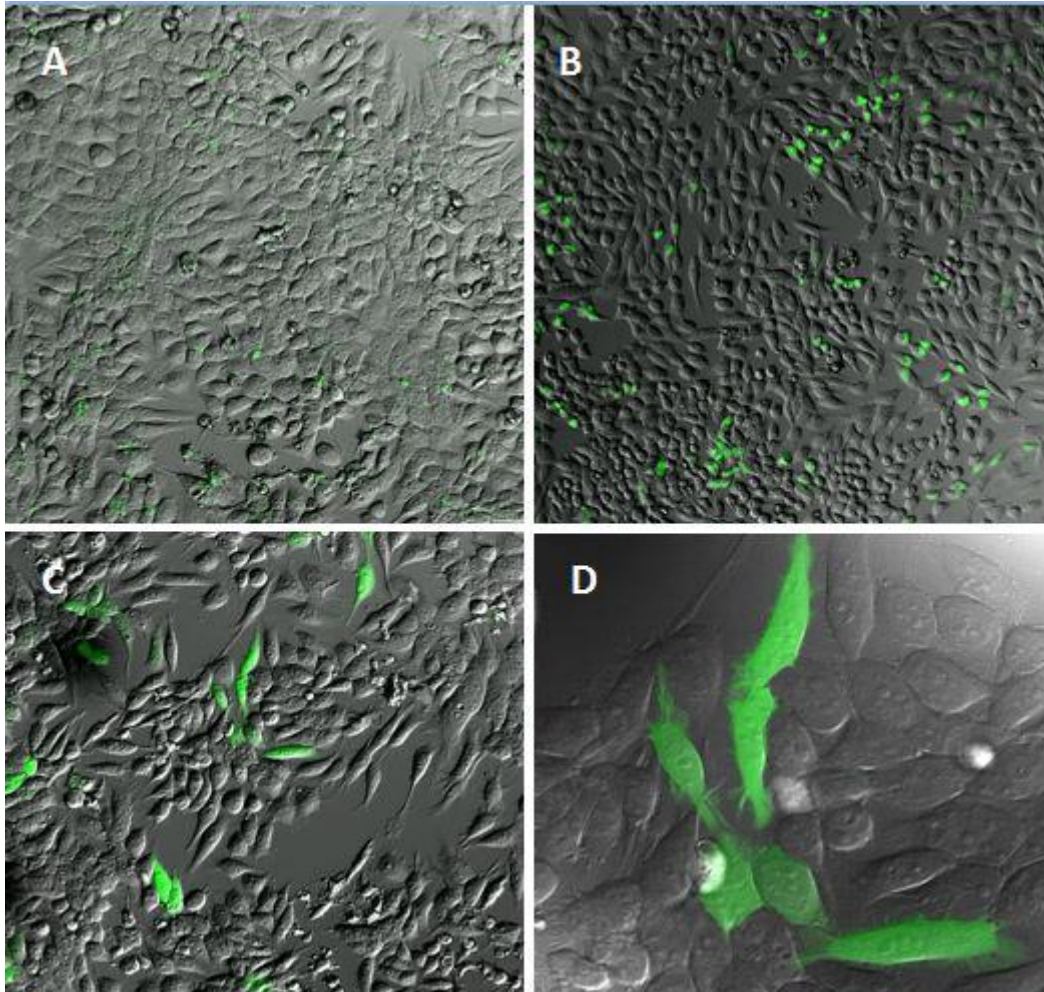


**Figure 7 - Confocal Microscopy of RY-HR HeLa Cells.** Stably integrated RY-HR HeLa cells were seeded over a cover slip and incubated for 24 hours. Following incubation, 5  $\mu\text{M}$  VP16 (**A**) and 100  $\mu\text{M}$  VP16 (**B**) was exposed to the cells for one hour. The cells were then washed with 1X PBS and incubated for 2 days. The cover slips were then removed and stained with a PI staining solution for 30 minutes. The cover slips were washed then adhered onto a slide using nail polish. Confocal microscopy was viewed with fluorescence for GFP and PI. (**A**) and (**B**) were captured at 20X magnification.

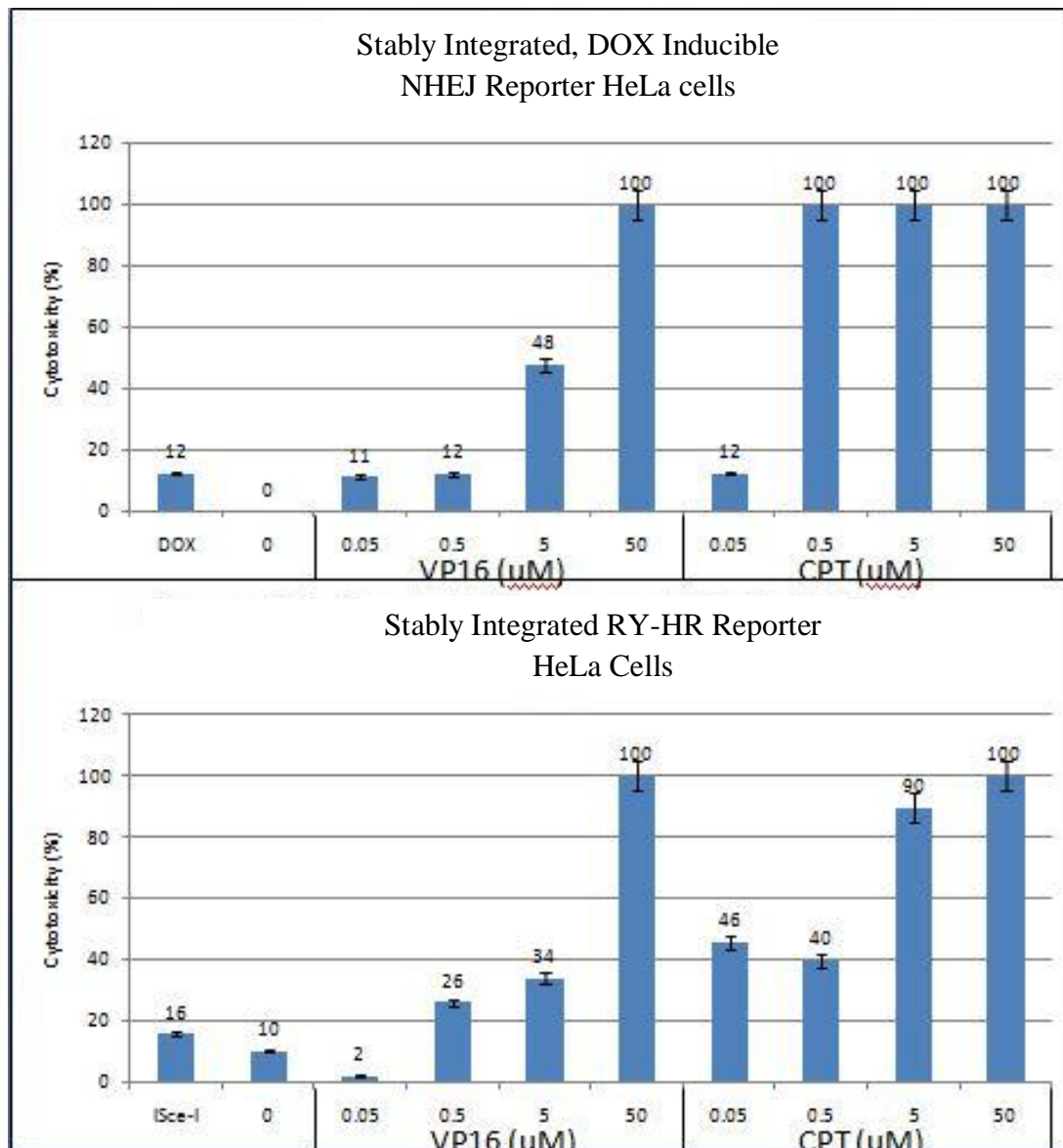


### *Confocal Microscopy*

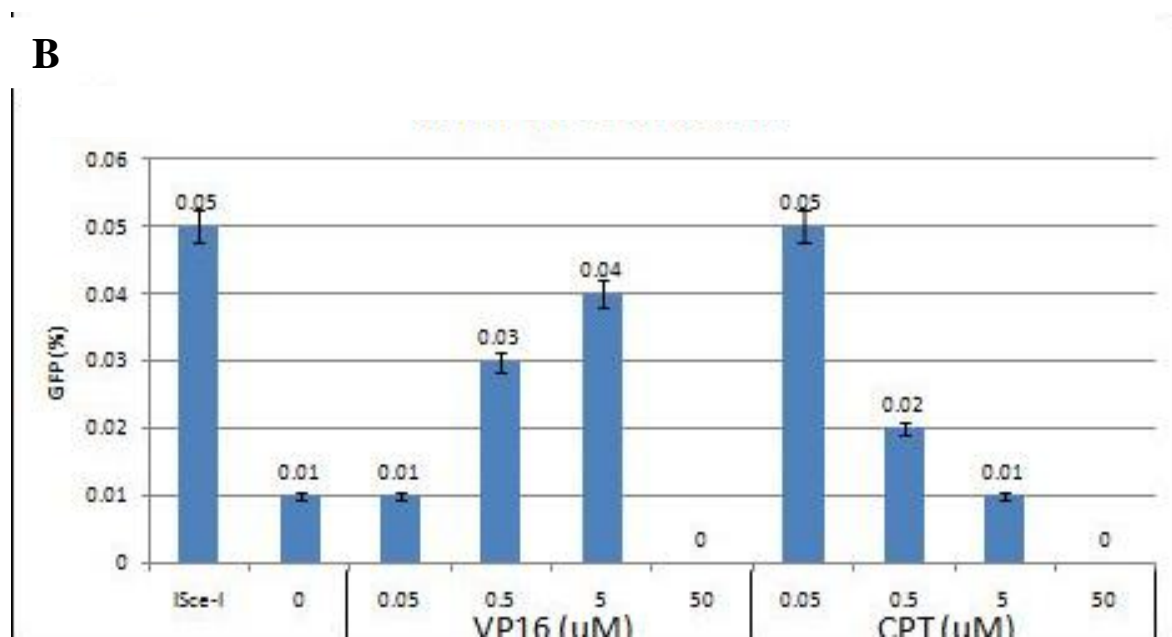
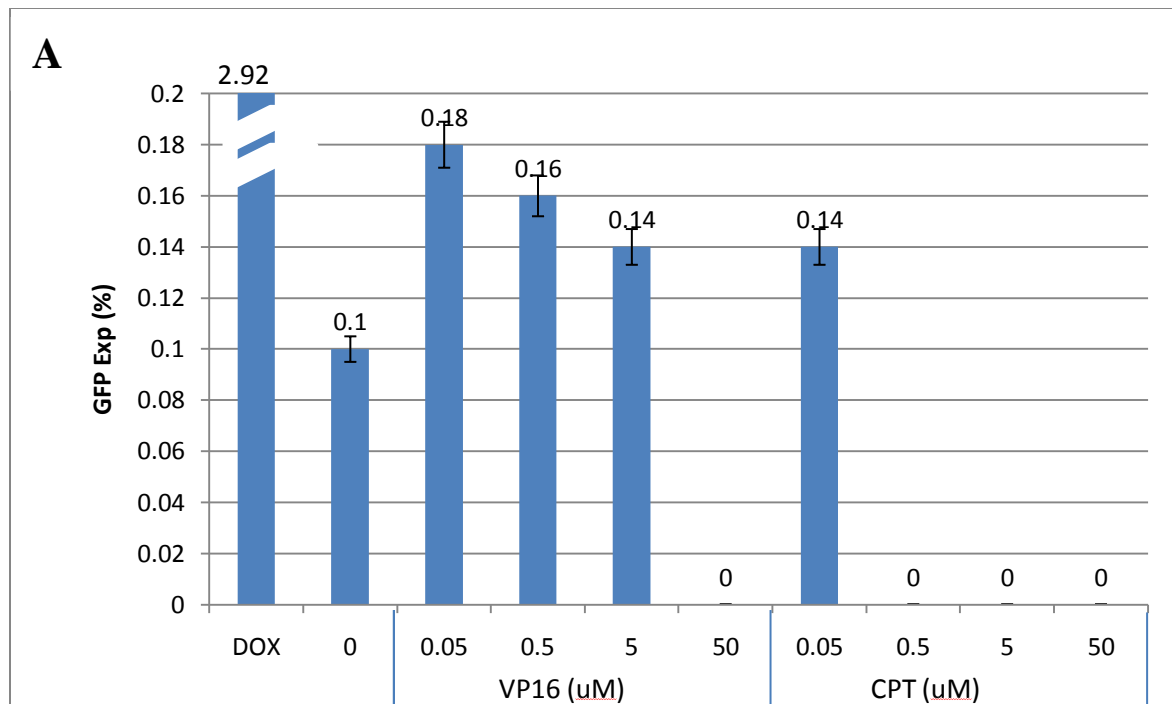
Confocal microscopy was performed to examine GFP expressing cells morphologically (**Figure 7 – 8**). The GFP protein appears to well distributed throughout the cell in the RY-HR cultures treated with VP16 for one hour followed by a 48 hour recovery interval. At 100  $\mu$ M VP16 there is an obvious toxic effect. Confocal analyses were performed on the doxycycline inducible NHEJ and HR reporter cells; see “Materials and Methods.” This experiment was preformed to examine any cytological differences between these two reporter systems. The NHEJ reporter yielded cells with GFP distributed throughout the cell (**Figure 8**). In contrast, the GFP produced as a result of HR in the HR clones appears largely in the nucleus, possibly due to a nuclear localization sequence on the GFP.



**Figure 8 - Confocal Microscopy of HR and NHEJ HeLa Cells.** Stably integrated doxycycline inducible NHEJ and HR HeLa cell lines were seeded at approximately 200,000 cells per well in a 12 well plate with an inherent slide base. Following 1 day incubation, doxycycline was added to the stated wells. The cells were incubated with the doxycycline for 3 days. (A) shows the uninduced HR HeLa cells at 20X zoom. (B) shows doxycycline induced HR HeLa cells at 20X zoom. (C) shows doxycycline induced NHEJ reporter system integrated HeLa cells at 20X zoom. (D) shows cells from (C) at 100X zoom. The confocal microscope was set to fluoresce solely GFP.



**Figure 9 - Cytotoxicity for NHEJ and RY-HR HeLa Cells.** Cytotoxicity percentages were calculated for stably integrated inducible NHEJ reporter systems in HeLa cells and stably integrated RY-HR HeLa cells. Both cultures were plated at 500,000 cells per well and incubated for 48 hours. Drug treatments lasted for one hour with both VP16 and CPT (TopoI Poison). The cells were then washed and allotted a recovery interval of 48 hours. Cytotoxicity percentages were calculated using trypan blue exclusion assays.



**Figure 10 - HR and NHEJ After VP16 Treatments** (A). illustrates GFP expression from doxycycline inducible stably integrated NHEJ reporter cassettes in HeLa cells. (B) shows stably integrated RY-HR HeLa cells. Both cell lines were plated at 500,000 cells per well and incubated for 48 hours. Drug treatments lasted for one hour for both VP16 and CPT (TopoI poison). For the positive controls; ISce-I transfection lasted for four hours and 3 day exposure for the inducible NHEJ system. The drug treated cells recovered for 3 days and were analyzed for GFP expression using FACS. Transfection efficiency was measured by pEGFP (18%).

### *Analysis of Toxicity*

The results of the trypan blue exclusion assay shows that with increasing concentration of VP16, there is an increase in cytotoxicity as expected (**Figure 9**). VP16 is a known chemotherapeutic and thus should have the high toxicity rating as shown in these results. CPT, a highly specific TopoI poison, shows an increased cytotoxicity amongst the NHEJ reporter system with concentrations from 0.5 – 50  $\mu$ M being toxic. The RY-HR HeLa cells show increased resistance to CPT. The RY-HR toxicity data does show that VP16 is generally less cytotoxic than CPT with regard to HR.

### *Analysis of Repair of Topoisomerase I and II DNA Damage by HR and NHEJ Pathways*

There are two major DSB repair pathways in animal cells; HR and NHEJ (see “Introduction”). Since we have dedicated reporters for each pathway and given that the drugs are highly specific for topoI (CPT) or topoII (VP16) mediated DNA damage, the repair process was evaluated in each case (**Figure 10**). Figure 10-A, the NHEJ reporter system, shows there is an increase in susceptibility in CPT damage and mild reparability in exposure to VP16 with dose dependent decrease in GFP. The positive control is noticeably elevated in comparison to the remaining samples. There is a decrease in NHEJ repair with an increase in VP16 concentration. Figure 10-B shows the effects of CPT and VP16 on RY-HR HeLa cells. There is an increase in HR with an increase in VP16 concentration and the opposite trend with CPT concentration. In both instances, 50  $\mu$ M of CPT or VP16 caused toxicity in the culture. The data points show that VP16 and CPT HR repair gives a GFP reading close to that of the positive control, ISce-I transfection.

## Chapter Four: Discussion

This project utilizes reporter cassettes to analyze the repair of DNA damage induced by topoII $\alpha$  and topoI poisons. Specific cassettes have been employed that report repair of DSBs by either HR or NHEJ (**Figure 4**). The first cassette, in the case of RY-HR cultures, contains the topoII $\alpha$  hot spot for the topoII $\alpha$  poison and/or an ISce-I restriction endonuclease cut site. The second cassette contains the template sequence to repair the induced DSB in the first cassette. Without drug treatment or transfection GFP is transcribed but not expressed due to mutations in the first sequence and a stop codon in the second sequence. The NHEJ pathway is being specifically measured using the NHEJ reporter (**Figure 4**) because the formation of wild type GFP cannot proceed by HR due to the lack of a homologous donor sequence.

The RY-HR GFP reporter systems indicate that with an increase in VP16 concentrations to 5  $\mu$ M (**Figure 10 - B**), there is an increase in HR repair. A dose dependent decrease in the NHEJ pathway (**Figure 10 - A**) in response to VP16 is also observed. The RY-HR integrated cells showed a dose dependent decrease in HR treated with CPT (**Figure 10-B**). The DSB initiated by VP16 appears to be repaired through the high fidelity, error free HR pathway. The high mitotic activity of cancer cells could make the cell cycle dependent HR repair pathway preferential compared to the non-cell cycle dependent NHEJ pathway. Most healthy cells are in the resting G1/G0 phase thus not prone to HR DSB repair.

The data in **Figure 9** define the toxicity profile of VP16. The highly specific topoI poison CPT had a higher toxicity than the VP16. HR may well be the preferred DSB repair pathway because the cancer cells are constantly dividing and HR is cell cycle specific. Moreover, HR proteins are associated with specific cell cycle checkpoints, whereas NHEJ is

not [14]. Due to the high mitotic levels of most cancer cells, HR should be a prominent pathway for break repair. However, not all cancer cell types follow the HR pathway preferentially. For example, both normal and malignant urothelial cells upregulate the NHEJ pathway, while HR is more dominant in cervical cancer cells [15]. The data and literature suggest that DSB repair pathways are highly variable and may well be dependent on tumor tissue location. Most importantly, since healthy cells preferentially use NHEJ to repair DSBs, a selective agent for HR proteins could sensitize cells to anti-cancer treatments while leaving healthy cells relatively unharmed. One report demonstrated that NHEJ targeting sensitized tumor cells and caused normal cell damage [16]. The targeting of HR could lessen the malevolent impact of chemotherapy on healthy cells, and thereby target cancer cells selectively.

Future work for this project includes analyzing, through the same reporter constructs, the effects of VP16 and CPT drug treatments on cell lines known for specific repair pathway upregulation. For instance, B cell lines are highly associated with NHEJ pathways. In addition, topoII $\beta$  analysis can be preformed based on the two pathways. TopoII $\beta$  has been found to be a causative enzyme of secondary malignancies in patients served topoII poisons. Finally, knockout or over expression of specific proteins upregulated in HR such as BRCA1 can be explored for its effects on both pathways via the GFP system.

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