



On-Treatment DNA Damage Response Biomarkers in Surrogate Tissue Types

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On-Treatment DNA Damage Response Biomarkers in
Surrogate Tissue Types

Katherine M. McDermott

A Thesis in the Field of Biotechnology

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Abstract

Given the challenges associated with collection of tumor specimens for biomarker monitoring, collection of surrogate tissues (e.g., normal tissues such as hair or blood) may enable the assessment of pharmacodynamic (PD) biomarkers. For chemotherapeutic agents, limited work has been done to establish pharmacodynamic (PD) biomarkers in these surrogate specimens. This study aims to demonstrate that surrogate specimens, specifically hair follicles and whole blood, are a viable option for assessment of PD biomarkers in response to chemotherapy in the clinic.

Traditional chemotherapeutic agents act by targeting rapidly dividing cells, one of the hallmarks of most cancers and some normal cells such as bone marrow, hair follicles and cells that line the digestive tract. Many chemotherapeutic agents, such as anthracyclines, topoisomerase inhibitors, and alkylating agents (e.g., platinum), work by directly damaging a cell's DNA in some or all phases of the cell cycle. The mechanism by which DNA damaging agents target erroneously dividing cells is indiscriminant and therefore results in DNA damage-induction in rapidly dividing healthy cells. The desire to better understand the biological reasons why cancer arises, propagates, and metastasizes, combined with a wider acceptance of personalized medicine, has led to the

development of targeted therapies. However, despite, the development of multiple anti-cancer targeted agents, chemotherapy remains the mainstay of cancer therapeutics. As such, there is a need to further develop biomarkers that aid in our understanding of chemotherapeutic activity.

In order to study these specimens, consent was obtained from healthy volunteers for the collection of hair follicles and whole blood, treated *ex vivo*, and evaluated by immunohistochemistry and flow cytometry for biomarkers of DNA damage. As an expansion of the whole blood work, blood was also obtained from rats after *in vivo* exposure to various DNA damaging agents.

This study demonstrates that human hair follicles are a viable option for biomarker monitoring in oncology. Specifically, γ H2AX can be used as a DNA damage marker. To enable robust assessment of DNA damage markers in surrogate specimens, multiple biological replicate must be collected given the expected variability in data generated. Results from this study also indicate that human whole blood will need more investigation before it is used clinically. Detection in whole blood was unsuccessful after *ex vivo* stimulation but *in vivo* exploration in rats yielded positive data.

Dedication

This thesis is dedicated to my husband, Sean, who encouraged and supported me to complete this degree. And to my daughter, Margaret, may the importance of education always be imparted on you.

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Chapter I

Introduction

One of the largest unmet medical needs in developed countries is the treatment of cancers. Cancer is a group of diseases characterized by the uncontrolled growth and spread of abnormal cells (Cancer Facts and Figures, 2014). Specifically, cancer cells have the ability to evade apoptosis, are self-sufficient in growth, are insensitive to anti-growth signals, metastasize and invade healthy tissues, have limitless replicative potential and promote angiogenesis (Hanahan et al., 2000). Over 1.6 million new cancer diagnoses are expected to occur in 2015, half a million of which are expected to be terminal diagnoses (Cancer Facts and Figures, 2014). Thus, cancer in general, illustrates a clear unmet medical need with much room for improvement in terms of treatments and therapies.

Standard of Care Therapies for the Treatment of Cancer

Surgery and radiation therapy dominated the cancer therapy space until the 1960s when the cure rates began to plateau at about 33% (DeVita, 2008). Chemotherapy agents, classified as a cancer treatment that uses chemical substances, were introduced shortly thereafter in the 1970's when adjuvant chemotherapy was shown to cure patients with

various advanced cancers. In adjuvant therapy, the chemotherapy is applied after the radiation and surgery to maximize effectiveness and ultimately suppress tumor formation. Adjuvant chemotherapy allowed physicians to target the cancerous cells leftover from surgery and radiation, thus leading to reduced metastases and significant improvements in efficacy. Since then, chemotherapy, which can be used as the primary therapeutic or in conjunction with surgery and radiation, has become the standard of care (SOC) for the treatment of a many tumor types (e.g., breast cancer, non-small cell lung cancer, pancreas cancer, ovarian cancer, etc).

Chemotherapy can be subdivided into several different classes based on the mechanism of action. Chemotherapy induces various types of DNA damage including modification of nucleotide bases, intrastrand crosslinks, interstrand crosslinks, DNA-protein crosslinks, single-strand breaks (SSBs), and double-strand breaks (DSBs). However, different chemotherapeutic agents have distinct mechanism of actions (MOAs). For the purposes of this study, we will focus on three classes of DNA damaging agents: alkylating agents (e.g., platinum), topoisomerase II inhibitors (e.g., etoposide and doxorubicin), and topoisomerase I inhibitors (e.g., irinotecan).

The chemotherapy drug carboplatin is classified as a platinum-based alkylating agent that is typically used to treat a wide range of cancers, most notably ovarian cancer. DNA alkylation causes DNA crosslinking and eventual single strand breaks (SSBs). This interferes with a cell's ability to repair DNA and/or create new DNA via replication. Interference with the cell's DNA repair machinery ultimately leads to cell death.

Another chemotherapeutic drug, etoposide, works in a different way by blocking the action of an enzyme called *topoisomerase II*. Etoposide is used to treat a wide range of different cancer types including testicular, bladder, prostate, lung, stomach and uterine cancers. Type II topoisomerase is responsible for cutting the strands of DNA, promoting chromosome disentanglement before strand separation during DNA replication. Blocking this enzyme leads to both single and double strand breaks in the DNA (SSBs, DSBs), and eventual cell death. Doxil is another *topoisomerase II* inhibitor that will be explored in this work.

Another mode of DNA damage is to target an enzyme called *topoisomerase I*. Chemotherapeutic agent, irinotecan, is a topoisomerase I inhibitor and is typically used to treat people with colon or rectal cancer. Similar to *topoisomerase II*, the *topoisomerase I* enzyme is responsible for breaking single-strand DNA to relax and reanneal the DNA strand. Inhibition of *topoisomerase I* causes both single (SSBs) and double strand (DSBs) breaks in the DNA, leading to cell death.

Since these chemotherapeutic drugs cannot distinguish between cancerous and non-cancerous cells, a fair amount of normal cell death occurs. This leads to undesirable side effects and toxicities in patients such as nausea, vomiting, hair loss, and diarrhea. However, chemotherapy works best on cells that are rapidly dividing which is a key characteristic of a cancer cell, making them more likely to be affected by chemotherapeutic agents. This ultimately allows for successful treatment outcomes albeit the undesired side effects.

Targeted Cancer Therapies

Standard of care (SOC) chemotherapy agents that are designed to kill cancer cells by inducing DNA damage are oftentimes coupled with specific, targeted, cancer therapies. This can lead to increased efficacy as the DNA damage, induced by the chemotherapeutic agent, is recognized and processed by proteins involved in the DNA damage response (DDR) such as PARP-1, DNA-PK, XRCC1, etc. Using targeted therapies to inhibit the DDR may enhance the therapeutic effect when combined with DNA-damaging agents (Hosoya, 2014). Preclinical studies have shown that combining PARP1 inhibitors with, for example, platinum chemotherapy agents, which induce DNA damage through adducts and cross linking, potentiates chemotherapeutic cytotoxicity, leading to better efficacy (Alli, 2009).

Careful monitoring of the PD effect of a targeted agent such as a PARP inhibitor requires an understanding of the PD effect in response to the chemotherapeutic alone. This will enable an understanding as to whether biomarker changes are due to the SOC treatment or targeted therapy. Understanding how DDR biomarkers change in response to DNA damaging agents may help inform the selection of a biologically active dose and/or dose schedule of combined therapies such as chemotherapy in combination with a PARP inhibitor. This study will aim to characterize the baseline pharmacodynamic biomarker effect DNA damaging agents before combination with targeted agents.

Biomarker Monitoring in Oncology

One commonly used definition of a biomarker is a measurable indicator that is used to precisely, reproducibly and objectively distinguish either a normal biological state from a pathological state, or the response to a specific therapeutic intervention (Gramont, 2014). Biomarker monitoring in the oncology setting is essential to gain a complete understanding of why a particular treatment may or may not be successful in the clinic. Within the broad field of oncology, there are *hundreds* of different types of cancers each with its own etiology, prognosis and course of treatment. In the development of most drugs in the oncology space two types of biomarkers tend to be utilized:

- 1) Patient selection markers
- 2) Target engagement/pharmacodynamics markers

Identification of patient selection markers is particularly important in cancer therapy given the heterogeneity of disease even among patients with the same diagnosis or cancer type. Patient selection biomarkers may begin to pull apart this heterogeneity and identify and treat subsets of patients that are predicted, based on their specific molecular defects and aspects of tumor microenvironment, to respond to a particular therapy (Kelloff, 2012). There are numerous well-known examples of targeted drugs and their respective biomarkers. For example, vemurafenib - a BRAF kinase inhibitor - has been approved for the treatment of metastatic melanoma patients who have the

BRAF^{V600E} mutation in their tumors. Patients that do not contain the BRAF^{V600E} mutation, are not predicted to respond to vemurafenib based on the MOA of the therapy and thus should not be treated or included in later phase clinical trials where you want to demonstrate clinical efficacy, but biomarker monitoring does not stop with this.

With an emphasis on molecularly targeted strategies, biomarkers that are indicative of a drug's pharmacodynamic mechanism of action or illustrative of target engagement are essential. Target engagement biomarkers can be proximal or distal, where the proximal biomarker is a direct target engagement readout and may even be evidence of a direct substrate of the target. For kinase drugs, for example, protein substrate phosphorylation represent candidate proximal target engagement biomarkers. However, substrate phosphorylation is oftentimes controlled by input from multiple converging pathways, complicating assessment of target engagement of a small molecule (Pawelcz, 2011). Thus, careful consideration and validation is needed when identifying candidate target engagement biomarkers as biomarker changes should be specific to modulation of the target of interest due to drug treatment. Distal target engagement biomarkers are generally located downstream of the target, are disease-related and are often referred to as outcome or pharmacodynamic biomarkers.

Pharmacodynamic (PD) biomarkers are modulated over time in response to a therapy and measure near-term treatment effects of a drug on the tumor or person (Sawyers, 2008). While pharmacokinetic (PK) measurements of drug concentrations in plasma are routine, establishing a relationship between the pharmacodynamic biomarker and pharmacokinetic profile provides a much deeper understanding of what is going on in

the patient after treatment with drug. Preclinical PK/PD profiling in xenograft models allows one to model the relationship between systemic drug exposure and biomarker changes associated with anti-tumor activity. This provides a benchmark for target PK exposures in the clinic as well as biomarker levels expected to render a clinically meaningful result (Takimoto, 2009). Furthermore, pharmacodynamic monitoring in the clinic can allow one to adjust dose and schedule based on the modulation of the PD biomarker to achieve the greatest coverage of target and ultimately efficacy.

Pharmacodynamic monitoring is complicated, however, by the combination treatment regimens oftentimes used in the oncology setting. Since combination therapy obscures the association between any one agent used in the treatment regimen and the biomarkers under consideration, it is important to understand the baseline response of certain biomarkers to SOC agents in order to fully appreciate modulation of the biomarker after treatment (Gramont, 2014).

Biomarkers of DNA damage

To ensure successful duplication and transmission of genetic material, the cell has evolved the ability to detect DNA damage which causes a variety of cellular responses including cell cycle arrest, DNA repair, senescence and apoptosis. Collectively, these processes are referred to as the DNA damage response (DDR). Damage, in the form of single strand or double strand DNA lesions, trigger activation of various kinases, most

importantly the phosphoinositide-3-kinase-related protein kinase (PI3K) family made up of ATM, ATR and DNA-PKcs.

When considering the frequency at which primary and adjuvant chemotherapy regimens are utilized, the majority of cancers at one point or another are treated with chemotherapy. There are a number of markers one could monitor in response to the DNA damage caused by chemotherapeutic agents. For purposes of this study, we will focus on three specific markers of DNA damage response: γ H2AX, pChk1, and pKAP1.

Double strand breaks (DSBs) are considered to be the most lethal forms of DNA damage since it prevents the cell from carrying out DNA replication. As such they must be identified and repaired quickly. As an early cellular response to DSBs, H2AX is phosphorylated at Ser-139 to produce γ H2AX. This phosphorylation event is now one of the most well-established chromatin modifications linked to DNA damage and repair (Mah, 2010). Phosphorylation of H2AX modulates DNA repair in three ways. First, γ H2AX facilitates DSB rejoining by anchoring the broken ends together through nucleosome repositioning. γ H2AX then recruits cohesin molecules that are protein complexes that regulate the separation of sister chromatids during cell division. The cohesins keeps the ends of the DNA strand in close proximity during repair, preventing large loss of chromosomal regions. Finally, γ H2AX modulates checkpoint responses, giving DNA time to repair (Mah, 2010). Thus, γ H2AX becomes a ubiquitous marker for DNA damage.

Ataxia telangiectasia and Rad3-related protein (ATR), one of the PI3K family proteins involved in the DNA damage response pathway, is activated upon recognition of DNA structures that are induced by DNA damage, such as single-stranded DNA (ssDNA) and junctions between ssDNA and double-stranded DNA (dsDNA) (Shiotani, 2009). In response to DNA damage, activated ATR phosphorylates Chk1 at Ser-345 which activates a cascade of DNA damage repair. Specifically, activation of Chk1 by phosphorylation arrests cells at the G2 phase of the cell cycle in response to DNA damage. As cells are damaged, pChk1 accumulates as an s-phase biomarker of cell synchrony in response to DNA damaging agents. Thus, pChk1 can be used to measure DNA damage caused by single strand breaks in the DNA.

PI3K family protein, ataxia telangiectasia mutated (ATM), becomes activated in response to DNA damage, specifically double strand DNA (dsDNA) breaks. KAP1 is a heterochromatin protein that is robustly phosphorylated by ATRM at Ser-824 in response to DNA damage and ATM activation. Therefore, pKAP1 can be used as a marker of DNA damage caused by double strand breaks in the DNA.

Table 1. Types of DNA damaging agents, the type of damage induced and biomarkers used for monitoring.

DNA Damaging agent	Category of DNA Damaging Agent	Type of Damage Induced	Biomarker for Monitoring
Carboplatin	Alkylating agent	Single-strand breaks in DNA (DSBs)	gH2AX; pChk1
Etoposide / Doxorubicin	Topoisomerase II inhibitor	Double-strand breaks in DNA (DSBs)	gH2AX; pKAP1
		Single-strand breaks in DNA (SSBs)	pChk1
Irinotecan	Topoisomerase I inhibitor	Double-strand breaks in DNA (DSBs)	gH2AX; pKAP1
		Single-strand breaks in DNA (SSBs)	pChk1

Tumor tissues for biomarker monitoring

Pharmacodynamic evaluation of targeted drugs using tumor biopsies obtained before and after drug exposure is usually performed in oncology trials to confirm that drug-induced biological effects achievable in preclinical models can also be observed in vivo (Ma, 2006). These effects may include modulation of a proximal target engagement marker, or pathway modulation of distal pharmacodynamic biomarkers. In a Phase II study using gefitinib, an orally active EGFR tyrosine kinase inhibitor used in patients with non-small-cell lung cancer (NSCLC), investigators aimed to determine antitumor activity of

gefitinib using sequential tumor biopsies obtained from patients on drug. Specifically, their goal was to study the effects of gefitinib in patients with advanced gastric cancer on EGFR phosphorylation and on the two major receptor signaling pathways - MAPK and PI3K/Akt, as well as its effect on proliferation and apoptosis (Rojo, 2006). Tumor biopsies were obtained at screening, prior to gefitinib treatment, on day 28 of treatment, and, if possible, at disease progression. Of the 70 patients enrolled on the study, only 32 (45.7%) had evaluable sequential paired biopsies prior to and post-gefitinib treatment. This speaks to the difficulty in procuring such an invasive specimen for research purposed only. This study highlights one of the challenges associated with collection of biopsies in clinical trials.

Analysis of these collected specimens becomes difficult as well when considering the heterogeneity of a tumor specimen. When considering analyzing the timed tumor biopsy specimen via an immunohistochemical (IHC) assay to assess a particular biomarker intratumorally, heterogeneity plays a major role in the likelihood of obtaining a meaningful result, especially when tumor material is so limited.

There are many additional challenges to successful incorporation of tumor tissue acquisition and analysis into clinical trial execution. For instance, a patient's malignancy and location of the proposed tumor biopsy play a significant role in the success of procuring the specimen. In a retrospective conducted by the NIH, six phase 1/2 clinical studies were evaluated to understand the optimal target tissue for safe research in tumor biopsies. Of the 142 patients that consented to research biopsies, 109 (77%) of them were ovarian cancer patients. By contrast, adrenal, mesothelioma, renal cell carcinoma and

thyroid cancer combined made up only 3% of the specimens analyzed (Lee, 2013). Thus, there is no doubt location and tumor type play a role in the likelihood of procuring these specimens.

In addition, there is risk of complication due to biopsy. For example, there is some concern around the “seeding” of tumor cells following biopsies. As the needle transgresses the tumor field and is withdrawn, there is potential for cells located in the tumor to migrate into the adjacent soft tissue and skin as a consequence of the violation of the tissue by the biopsy needle (Loughran, 2011). Furthermore, in a study that assessed a total of 57 clinical trials, biopsy-related complications were observed in 5.2% (39 of 745 biopsies) (Overman, 2013). Complications were related to the location of the biopsy, with the highest complication rate being for intrathoracic biopsies at 17.1% (Overman, 2013).

Complications due to biopsy and general inconvenience may lead to decreased consent to optional research assessments. This may make biomarker monitoring in the clinic difficult if target engagement or pharmacodynamic effect cannot be confirmed. Thus, there needs to be alternative ways to monitor disease effect via biomarker in the clinic besides assaying the actual tumor repeatedly.

Surrogate tissues for biomarker monitoring

While tumor biopsies are considered to be the gold standard for assessing molecular changes in tissue to guide drug development in oncology, there is an increasing uptake of the use of normal “surrogate” tissues into clinical trials. Biomarker profiling in surrogate tissues, such as blood or hair follicles, offer many advantages over the invasive sampling of tumor tissue. For example, collections of surrogate tissues incur low procedural risk, as collections are far less invasive. As such, there is a high likelihood a patient will consent to the sampling leading to increased compliance and a more complete understanding of the markers evaluated in the surrogate specimens.

Furthermore, surrogate specimens are amenable to repeat sampling in a way tumor biopsies generally are not. Instead of only taking a pre- and post-treatment sample, surrogate specimens can be sampled many times over a period of time. This allows for full characterization of pharmacodynamic changes that occur in response to drug treatment.

PBMCs or peripheral blood mononuclear cells are the populations of immune cells that are oftentimes extracted from a whole blood sample to perform biomarker analyses on. PBMCs include lymphocytes, monocytes and dendritic cells and are characterized as having a round nucleus. As such, they can be used to monitor gene expression in whole blood, making them amenable to biomarker monitoring. During development of a first-in-class hsp90 inhibitor, the correlation between pharmacodynamic changes in PBMCs and in tumor tissue was established in vivo models. Following this, in

the PK/PD driven Phase I study tumor biopsies were performed only after the satisfactory demonstration of plasma concentrations above those required for activity in human tumor xenograft models and evidence of pharmacodynamic modulation in PBMCs (Ang, 2012). PBMCs were also used to develop a PK/PD model for Everolimus, an mTOR inhibitor that was recently approved by the FDA and EMA. Everolimus has been demonstrated to inhibit the phosphorylation and activity of the downstream mTOR-regulated SK6 in vitro, in vivo in tumors as well as in surrogate tissues in both rats and mice (O'Reilly, 2010). As a result, detection of S6K1 activity in human PBMCs was used to evaluate everolimus dosing schedules. Specifically, S6K1 activity was monitored in PBMCs derived from healthy human volunteers after ex vivo treatment with 2nM everolimus for 30 minutes leading to 44% and 63% inhibition in two different donors (O'Reilly, 2010). Exploration of dose and schedule using PK/PD modeling in human PBMCs allows for exploration of a sufficient drug level to inhibit a biological endpoint to provide an optimal biologic dose. These two studies, among others, demonstrate the utility of PBMCs in the biomarker space.

Hair follicles offer another attractive surrogate specimen that is sometimes used in pharmacodynamic biomarker monitoring in the clinic as evidence of the mode of action of the drug on human tissues via the proposed pathway. Plucked human hairs contain proliferating cells within the hair sheath making them attractive as an easily accessible tissue in which to assess the pharmacodynamic effects of drugs that interfere with cell proliferation, such as chemotherapeutic agents (Camidge, 2005). In addition, hair follicles are highly vascularized, making them susceptible to similar drug exposures seen in the

plasma. Methods for the fixation, processing, sectioning and immunohistochemical (IHC) staining of a number of oncology-relevant antibodies on plucked hair has been previously described and summarized by Randall, et al. (2007). Similar methods have been utilized in hair follicles by Yap, et al. (2010) to develop novel biomarker assays for the inhibition of AKT in human hair follicles in the clinic. Specifically, phosphorylation and total protein expression of the AKT substrate PRAS40 was measured in hair follicles. Significant decreases in pThr246 PRAS40 was demonstrated in AKTi treated mouse whisker follicles *in vivo* and human hair follicles treated *ex vivo*, with minimal changes in total PRAS40 (Yap, 2010).

Given the above considerations in surrogate tissues, we propose *ex vivo* evaluation of surrogate specimens for tumor tissue that can be used to perform biomarker analyses, specifically in hair follicles and peripheral blood mononuclear cells (PBMCs). By studying DNA damage biomarkers in surrogate tissues, such as hair follicles and blood, it will help us understand how these surrogate tissue specimens respond to traditional DNA damaging agents and whether or not they can be used for biomarker monitoring in the clinic.

Chapter II

Materials and Methods

A variety of methods were employed during this research to examine biomarkers of DNA damage in human hair follicles and human whole blood. In order to study these specimens, consent was obtained from healthy volunteers for the collection of hair follicles and whole blood as described below and treated *ex vivo*. As an expansion of the whole blood work, blood was also obtained from rats after *in vivo* exposure to various DNA damaging agents. Both immunohistochemistry and flow cytometry were utilized to examine the prepared specimens. Methods described in more detail as follows.

Biospecimen Collection

Human scalp hairs were plucked with tweezers from the side of the head and visually checked for the presence of an anagen hair bulb (growth phase). The hair bulbs were then transferred to either 10% buffered formalin for immediate fixation, cell culture media (proprietary, provided by Epistem LTD.), or cell culture media supplemented DNA damaging agents (e.g. Cisplatin, Etoposide, Irinotecan (SN38)). A total of 5 anagen hair follicles were collected per treatment group and cultured for 17, 24, or 28 hours,

excluding the hairs that were uncultured and fixed immediately. Specific culture conditions and processing described below.

Human whole blood was collected intravenously into 10mL sodium heparin (NaHep) Vacutainer® tubes (BD, Catalog number: 366480) and stored at room temperature overnight before processing. Blood was aliquoted and treated with DNA damaging agents (e.g. Carboplatin, Etoposide, Irinotecan (SN38)) for 1, 4, and 8 hours. Specific treatment conditions described below.

Whole blood from rats treated with Etoposide was collected at 1, 2, 7 and 24 hours post dose. Specifically, rats were cannulated in jugular vein and blood collection was from cannula. Blood was immediately lysed and fixed.

DNA Damage Induction in Hair

To assess known DNA damage markers in hair follicles, hair follicles were collected from three healthy male donors and immediately transferred to ex vivo culture conditions according to Table 2 below.

Table 2. Experimental groups to assess known DNA damage markers in hair follicles.

Experimental Group	Treatment	Harvest time	Number of hairs / donor	Number of Sections analyzed / hair	DNA damage maker analyzed
1	NA- Uncultured fresh plucked hairs	Fixed Immediately	5	3	γ -H2AX, pChk1
2	Untreated	24 hours	5	3	γ -H2AX, pChk1
3	Vehicle (0.1% DMSO)	24 hours	5	3	γ -H2AX, pChk1
4	50 μ M Carboplatin	24 hours	5	3	γ -H2AX, pChk1
5	10 μ M Etoposide	24 hours	5	3	γ -H2AX, pChk1
6	100nM Etoposide	24 hours	5	3	γ -H2AX, pChk1

To assess the dynamics of DNA damage in hair, hair follicles were collected from three healthy male donors and immediately transferred to an ex vivo culture and harvested at multiple timepoints according to Table 3 below.

Table 3. Experimental groups to assess biomarker dynamics at multiple timepoints.

Experimental Group	Treatment	Harvest time	Number of hairs / donor	Number of Sections analyzed / hair	DNA damage maker analyzed
1	NA- Uncultured fresh plucked hairs	Fixed Immediately	5	3	γ -H2AX
			5	4	pChk1
2	Untreated	17, 24, 28 hours	5	3	γ -H2AX
			5	4	pChk1
3	Vehicle (0.1% DMSO)	17, 24, 28 hours	5	3	γ -H2AX
			5	4	pChk1
4	50 μ M Carboplatin	17, 24, 28 hours	5	3	γ -H2AX
			5	4	pChk1
5	10 μ M Etoposide	17, 24, 28 hours	5	3	γ -H2AX
			5	4	pChk1
6	100nM Etoposide	17, 24, 28 hours	5	3	γ -H2AX
			5	4	pChk1

Hair follicles were cultured in cell culture media (provided by Epistem LTD., proprietary) or cell culture media supplemented with Carboplatin (Sigma, Catalog number: C2538), Etoposide (Sigma, Catalog number: E1383), or SN38, the active metabolite of Irinotecan (Santa Cruz, Catalog number: sc-203697) according to Table 2

and Table 3. Specifically, Carboplatin was prepared as a 50mM stock solution in DMSO by dissolving 100mg of Carboplatin into 5.387mL DMSO. The stock was then further diluted 1:1000 into proprietary cell culture media producing final working concentration of 50uM (0.1% DMSO volume).

Etoposide was prepared as a 20mM stock solution in DMSO by dissolving 58.856mg of Etoposide into 5mL DMSO. The stock was then further diluted 1:2000 into proprietary cell culture media producing a final working concentration of 10uM (0.05% DMSO volume).

SN38, which is the active metabolite of Irinotecan, was prepared as a 200uM stock solution in DMSO by dissolving 10mg of SN38 into 25.486mL DMSO (1mM stock) and then a further serial dilution (1:5) again into DMSO. The 200uM stock was finally diluted 1:2000 into proprietary cell culture media producing a final working concentration of 100nM (0.05% DMSO volume).

All concentrations of DNA damaging agents were chosen as they were shown to kill a large number of cancer cells in *in vitro* experiments (data not shown) and were determined to be the IC90 concentrations in the particular assays utilized.

Processing of Plucked Human Hairs

After culture, or immediately upon collection, hairs were fixed in 10% buffered formalin for 30 minutes and transferred to 70% ethanol for up to a week for paraffin embedding (one hair per paraffin block). Ten longitudinal 3 μ M thick sections were cut per block per donor per experimental group. Sections were mounted onto charged slides and individual sections were stained for γ -H2AX and pChk1 and analyzed by IHC.

Immunohistochemistry

Slides containing hair sections were de-waxed in xylene and rehydrated through graded alcohols to PBS. Endogenous peroxidase was blocked with 0.3% H₂O₂ in PBS before rinsing in PBS. Non-specific binding was blocked with 10% normal goat serum in TBST for 30 minutes.

After removing blocking serum, the sections were incubated with a rabbit polyclonal anti- γ -H2AX antibody (Bethyl Laboratories, Catalog number: IHC-00059) at a dilution of 1:250 in TBST containing 5% normal goat serum and incubated for 1 hour at room temperature. Sections were then rinsed with TBST and detection of primary antibody was carried out by using a biotinylated goat-anti-rabbit secondary antibody (Vector Lab Inc, diluted 1:200 for 30 minutes) followed by incubation for 30 minutes

with Vector ABC Elite reagents (Vector Lab Inc.; peroxidase-labeled avidin-biotin complex).

To evaluate pChk1 staining, sections underwent antigen retrieval by microwaving for 20 minutes in pH6 citrate buffer before blocking endogenous peroxidase using 3% H₂O₂ in RO water for 10 minutes. Non-specific binding was blocked using 5% normal goat serum in PBST for 20 minutes. Sections were then incubated with rabbit monoclonal anti-pChk1(ser345) antibody (Cell Signaling, Catalog number: 2348) diluted 1:50 in PBST for 1 hour at room temperature. Sections were subsequently rinsed in PBST and detection of the primary antibody was completed using biotinylated goat-anti-rabbit secondary antibody (Vector Lab Inc, diluted 1:200 for 30 minutes). This was rinsed in PBST and all slides were treated for 30 minutes using Vector ABC Elite reagents (Vector Lab Inc.; peroxidase-labeled avidin-biotin complex).

For both gH2AX and pChk1 staining, primary antibody was removed and substituted with TBST as a negative control. Within each labeling run, two slides of skin biopsies (+/- UVB induced DNA damage) was used as a positive and negative controls, respectively. Labeling was visualized with ImmPAT DAB peroxidase substrate (Vector Lab Inc). All sections were finally counterstained using Garvey's haematoxylin and dehydrated through to xylene.

Skin tissues were treated with 0.485 J/cm² UVB irradiation to be used as a positive control for induction of both gH2AX. OD26749 xenograft tumor tissues treated

with 3mpk Cisplatin were used as positive controls for pChk1 labeling after induction of pChk1 was confirmed via western blot.

Quantification of Labeling in Plucked Hair Sections

Stained hair sections were scanned using the Aperio® image analysis platform and the epithelial outer root sheath (ORS) regions were selected for analysis. Thresholds were manually calibrated to group positive cells into distinct labeling intensity groupings. Specifically, for gH2AX labeling, 1+ or weak staining was identified as staining with thresholds between 195 and 181. 2+ or moderate staining was identified as staining with thresholds between 180 and 151. 3+ or strong staining was identified as staining with thresholds 150 to 0. Due to the small number of pChk1 positive cells and lack of strong intensity variation, all positive cells for pChk1 were identified as staining with thresholds from 206 to 0.

DNA Damage Induction in Blood

Human whole blood was aliquoted, in duplicate, into two 15mL conicals and treated with compound at varying concentrations to give a total volume of 100ul. Specifically, Carboplatin (Sigma, Catalog number: C2538) was prepared as a 50mM

stock by dissolving 100mg Carboplatin into 5.38mL DMSO. The 50mM stock solution was diluted 1:100 in PBS to create a 500uM working stock. The 500uM working stock was finally diluted 1:10 in whole blood producing a final working concentration of 50uM (0.1% DMSO).

Etoposide was prepared as a 50nM stock by dissolving 100mg Etoposide (Sigma, Catalog number: E1383) into 3.4mL DMSO. A 1:400 dilution in DMSO followed by a 1:100 dilution in PBS resulted in a 100uM working stock. The 100uM working stock was finally diluted 1:10 in whole blood producing a final working concentration of 10uM (0.1% DMSO).

SN38, the active metabolite of Irinotecan, (Santa Cruz, Catalog number: sc-203697) was prepared as a 10mM stock by dissolving 10mg SN38 into 2.55mL DMSO. A working stock of 1uM was created by diluting the 10mM stock in DMSO by four 1:10 serial dilutions in DMSO where the last dilution was in PBS. The 1uM working stock was finally diluted 1:10 in whole blood producing a final working concentration of 100nM (0.1% DMSO).

After the blood was treated with compound, tubes were shaken to ensure mixing and placed in a 37°C 5% CO₂ incubator for 1, 4, and 8 hours. As a positive control, additional aliquots of blood were placed into a γ -particle irradiator and received 2 Gy of radiation from a cesium-137 source and were placed in a 37°C 5% CO₂ incubator for 1, 4, and 8 hours.

After the treatment duration, 50X volume of Phosflow™ (1X concentration, warmed to 37°C) (BD, Catalog Number: 554656) was added to the blood samples to lyse/fix the samples. Samples were inverted 8-10 times to ensure mixing and incubated at room temperature for 10 minutes to ensure complete cellular lysis. Tubes were spun at 200 x g for 10 minutes and supernatant was discarded. 200ul PBS was added to each pellet and samples were pipetted up and down to ensure mixing. Samples were transferred to a 96-well v-bottom plate and plates were spun at 2000rpm for 5 minutes. Supernatant was removed and 200uL ice cold 90% methanol was added and pipetted up and down to ensure mixing. Plates were covered with a plate cover and allowed to permeabilize overnight at -20°C.

After permeabilization, samples were pipetted up and down to ensure mixing and 100ul of each sample was transferred to a new plate and the remaining material was stored at -80°C. Plates were spun at 2000rpm for 5 minutes and the supernatant was removed. 20ul staining master mix containing 5ul gH2AX antibody (BD Biosciences, anti-H2AX (pS139), Alexa Fluor 647, Clone: N1-431, Catalog # 560447), 0.5ul pKAP antibody (Pierce, Phospho-TIF1beta, pSer824 Ab DyLight 488, Lot #PL212965), and 14.5ul PBS-0.5% BSA was added to each sample and was incubated at room temperature for 1 hour, on a plate shaker. 200ul PBS + 0.5% BSA was added to each sample and the plates were spun at 2000rpm for 5 minutes. Supernatant was discarded. Samples were resuspended in 100ul PBS + 0.5% BSA and evaluated via flow cytometry as described below.

DNA Damage Induction In Vivo

To explore the in vivo response to DNA damaging agents, rats were given an intraperitoneal (ip) injection of 10 or 15mg/kg etoposide. Radiation (2Gy) and DMSO treated animals were used as controls. All treatment groups contained quadruplicate replicates. Rats were cannulated in jugular vein and blood collection was from cannula. Whole blood was collected at 1, 2, 7 and 24 hours post dose and fixed immediately by adding 50X volume of Phosflow™ (1X concentration, warmed to 37°C) (BD, Catalog Number: 554656).. Samples were inverted 8-10 times to ensure mixing and incubated at room temperature for 10 minutes to ensure complete cellular lysis. Tubes were spun at 200 x g for 10 minutes and supernatant was discarded. 200ul PBS was added to each pellet and samples were pipetted up and down to ensure mixing. Samples were transferred to a 96-well v-bottom plate and plates were spun at 2000rpm for 5 minutes. Supernatant was removed and 200uL ice cold 90% methanol was added and pipetted up and down to ensure mixing. Plates were covered with a plate cover and allowed to permeabilize overnight at -20°C.

After permeabilization, samples were pipetted up and down to ensure mixing and 100ul of each sample was transferred to a new plate and the remaining material was stored at -80°C. Plates were spun at 2000rpm for 5 minutes and the supernatant was removed. 20ul staining master mix containing 5ul gH2AX antibody (BD Biosciences, anti-H2AX (pS139), Alexa Fluor 647, Clone: N1-431, Catalog # 560447), 0.5ul pKAP antibody (Pierce, Phospho-TIF1beta, pSer824 Ab DyLight 488, Lot #PL212965), and

14.5ul PBS-0.5% BSA was added to each sample and was incubated at room temperature for 1 hour, on a plate shaker. 200ul PBS + 0.5% BSA was added to each sample and the plates were spun at 2000rpm for 5 minutes. Supernatant was discarded. Samples were resuspended in 100ul PBS + 0.5% BSA and evaluated via flow cytometry as described below.

Flow Cytometry

The 96-well plates were loaded onto the BD FACSCalibur™ platform and the flow rate was set to 35uL/min. Acquisition was gated based on the lymphocyte population and was set to collect data until 1 million events were captured. The number of cells positive for both pKAP1 and pH2AX were captured.

Statistical Analyses

For the hair follicle and flow cytometry assays, all data were plotted using Microsoft Excel 2011 (Microsoft). Data from these two assays were normalized to vehicle controls and two-tailed T tests were performed to determine whether DNA damage marker (e.g. gH2AX, pChk1, pKAP1) expression significantly differed between donors and treatment groups.

A statistical analysis of pChk1 labeling from the 24-hour experiment was performed using a linear random effects model to try to understand, from the observed variability, how many hairs would need to be analyzed to see a statistically significant change in pChk1 expression.

Chapter III

Results

This study demonstrates that human hair follicles are a viable option for biomarker monitoring in oncology. Specifically, gH2AX can be used as a DNA damage marker. However, the number of hair follicles collected must be carefully considered before incorporating this collection into clinical studies due to the variability in this sample type. Results from this study also indicate that human whole blood will need more investigation before it is used clinically. Detection in whole blood was unsuccessful after ex vivo stimulation but in vivo exploration in rats yielded positive data indicating that there may be a disconnect between the ability to fully detect damage induction in ex vivo and in vivo experiments.

Evaluation of DNA Damage in Formalin Fixed Paraffin Embedded Hair Follicles via Immunohistochemistry

In order to evaluate DNA damage response in hair follicles, hairs were plucked from volunteer subjects in accordance with the protocol. Plucked follicles were treated with three DNA damaging agents: carboplatin, etoposide, and irinotecan. Hair follicles were then formalin fixed and paraffin embedded in preparation for assessment of DNA damage markers by immunohistochemistry (IHC). Hair follicles sectioned for IHC

evaluation were incubated with anti- γ -H2AX antibody at a range of dilutions from 1:50 to 1:500 in TBST containing 5% normal goat serum in order to optimize labeling. Following primary antibody incubation, samples were incubated with biotinylated goat-anti-rabbit IgG (Vector BA-1000) at 1:200 for 30 minutes at room temperature. After evaluation of the range of primary antibody concentrations, the 1:250 dilution was chosen as optimal. This staining protocol was utilized throughout the rest of the study. Figure 1 below illustrates a representative hair follicle stained with anti- γ -H2AX antibody at 1:250 dilution after treatment with etoposide.



Figure 1. γ H2AX labeling following etoposide treatment. Image taken using 10X objective. Brown = DAB

Similarly, hair follicles sectioned for IHC evaluation were incubated with anti-pChk1 (Ser345) antibody at a range of dilutions - 1:50, 1:100, 1:200, 1:500 and 1:1000 in TBST containing 5% normal goat serum in order to optimize labeling. Following primary antibody incubation, samples were incubated with biotinylated goat-anti-rabbit IgG (Vector BA-1000) at 1:200 for 30 minutes at room temperature. After evaluation of the

range of primary antibody concentrations, the 1:50 dilution was chosen as optimal. This staining protocol was utilized throughout the rest of the study. Figure 2 below illustrates a representative hair follicle stained with anti-pChk1(ser345) antibody at 1:50 dilution after treatment with carboplatin.

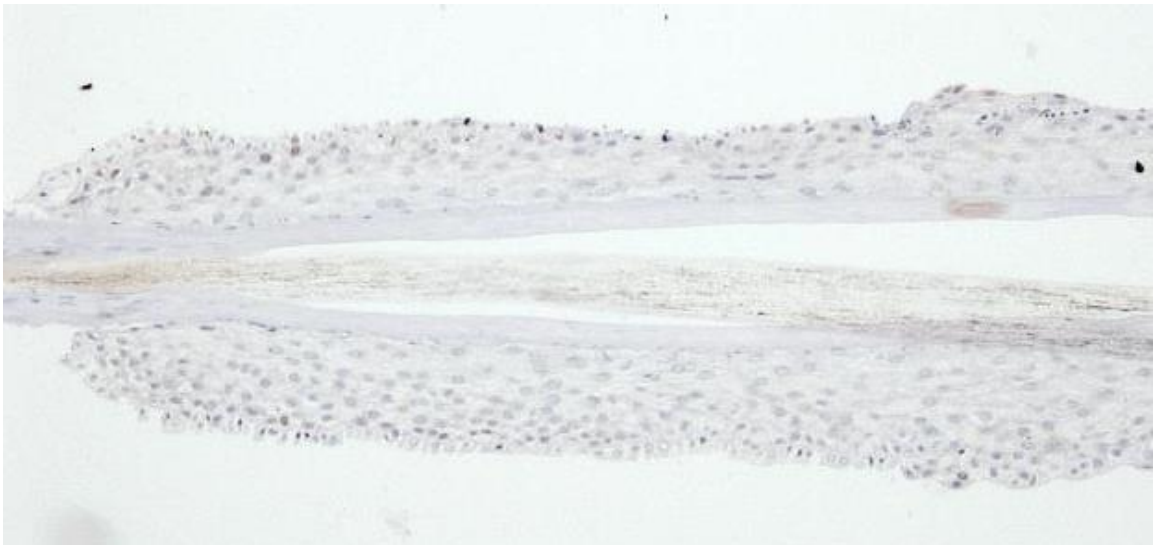


Figure 2. pChk1 labeling following etoposide treatment. Image taken using 10X objective. Brown = DAB

DNA Damage Induction in Response to Carboplatin or Etoposide in Hair Follicles as Measured by γ -H2AX and pChk1

To assess the variability in γ -H2AX and pChk1 induction, mean scores for cumulative total percent positive cells were calculated for both γ -H2AX and pChk1 after treatment with DNA damaging agents. Standard deviation was determined using the total percent positive scores between hairs within a group. Donor 1 demonstrated an increase

in the percent positive nuclei for γ -H2AX across all three test items when compared to DMSO alone. Two tailed Student's T-tests applied to the data showed that the induction of γ -H2AX after treatment with 10 μ M etoposide was significant ($p=0.006$) compared to the DMSO control group. Conversely, despite the increases in γ -H2AX staining with 50 μ M carboplatin and 100nM SN38, both were found to be insignificant ($p=0.078$ and $p=0.419$, respectively). Results are presented in Figure 3.

Donor 2 showed only a slight increase in the number of labeled cells when cultured *ex vivo*. Only treatment with etoposide induced a significant increase in the number of positively labeled nuclei for γ -H2AX compared to DMSO control ($p=2.9 \times 10^{-5}$). While *ex vivo* treatment with carboplatin induced an increase in γ -H2AX positive nuclei, the induction was not statistically significant when compared to DMSO control ($p=0.15$). Conversely, treatment with SN38 resulted in a decrease in γ -H2AX expression, however this was insignificant ($p=0.12$). Results are presented in Figure 3.

Carboplatin, etoposide and SN38 all showed increased expression of γ -H2AX in comparison to DMSO in Donor 3. A two-tailed T-test proved the increase due to treatment with both carboplatin and etoposide was statistically significant when compared to DMSO controls ($p=0.03$ and 0.0014 , respectively). One of the three donor 3 hair follicles sectioned and stained after treatment with etoposide was excluded from analyses due to poor quality. The change in labeling expression for SN38 was not significant ($p=0.10$). Results are presented in Figure 3.

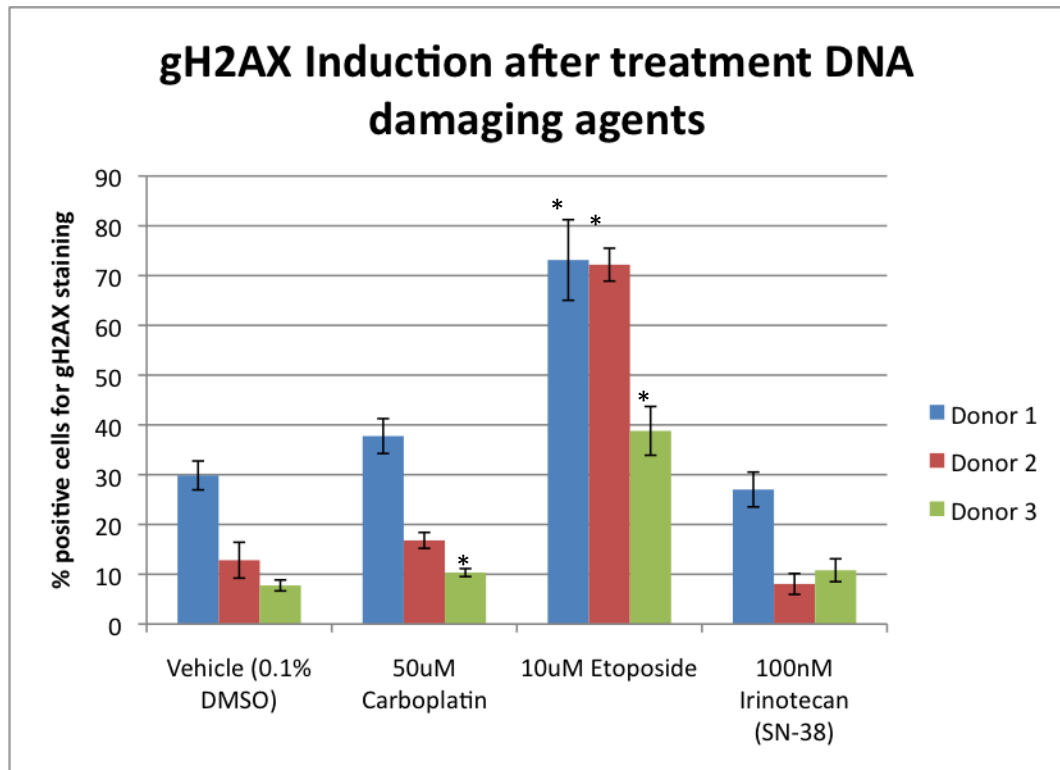


Figure 3. Expression of gH2AX after 24 hours of ex vivo treatment with DNA damaging agents. Percent positive cells for gH2AX after treatment with carboplatin, etoposide and SN38 normalized to DMSO control. * denotes significance (two-tailed T-test against equivalent donor vehicle; $p < 0.05$)

Overall, the variability in gH2AX induction between hair follicles from a single donor was high across all donors. Further, all DNA damaging agents lead to an increase in damage as measured by gH2AX with etoposide yielding the most robust and consistent response across all three donors.

In donor 1, all three DNA damaging agents resulted in an increase in the percentage nuclei positively labeled for pChk1 compared to DMSO. However the only significant increase in pChk1 labeling compared to DMSO was after ex vivo treatment

with etoposide ($p=0.02$). Treatment with carboplatin and SN38 resulted in non-significant increases in pChk1 ($p=0.37$, $p=0.12$, respectively). Results are presented in Figure 4.

In donor 2, treatment with carboplatin resulted in a slight increase when compared to vehicle, with much larger inductions in pChk1 labeling seen after treatment with both etoposide and SN38. Treatment with etoposide was the only agent to induce a statistically significant increase in pChk1 expression ($p=0.03$). Treatment with carboplatin and SN38 resulted in insignificant changes in pChk1 ($p>0.05$). Results are presented in Figure 4.

Donor 3 showed much lower pChk1 labeling than donors 1 and 2 but demonstrated small increases in pChk1 expression following exposure to DNA damaging items (all insignificant). Results are presented in Figure 4.

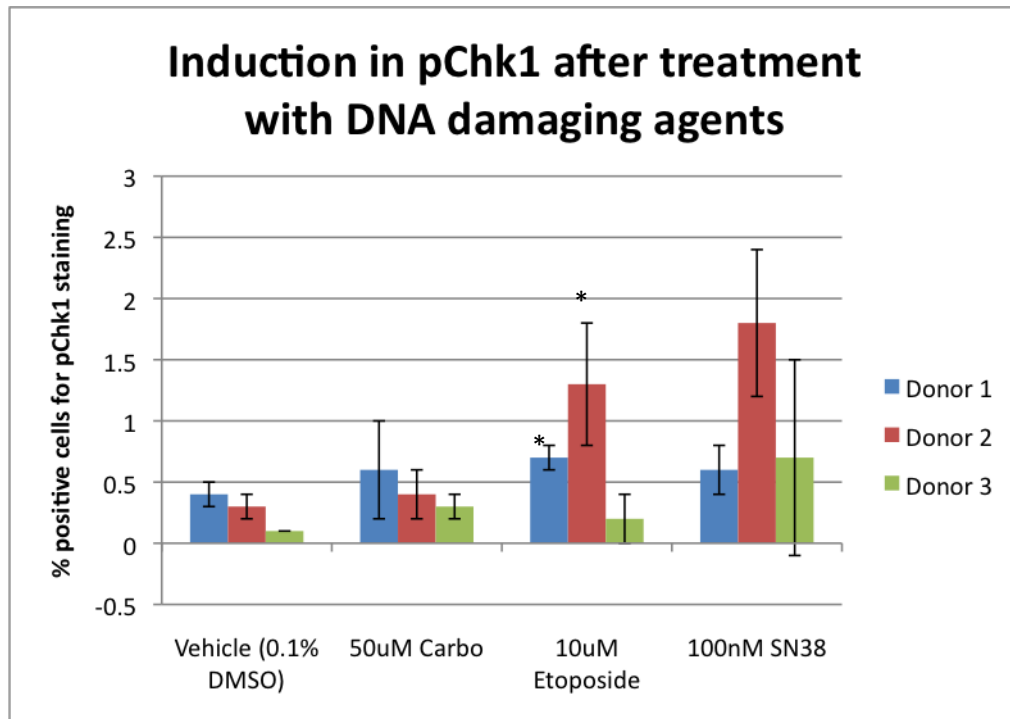


Figure 4. Expression of pChk1 after ex vivo treatment with DNA damaging agents. Percent positive cells for pChk1 after treatment with carboplatin, etoposide and SN38 normalized to DMSO control. * denotes significance (two-tailed T-test against equivalent donor vehicle; $p < 0.05$)

Overall, the variability between hair follicles from a single donor was high across all donors and DNA damaging agents, but the trend (increased DNA damage) was consistent across the group of three donors. Given the expected variability, more hair follicles from individual donors may be required to drive a more accurate assessment of pChk1 induction. Also of note, the total number of cells staining positive for pChk1 was extremely low with treatment with etoposide yielding the most robust response in DNA damage, as measured by pChk1 with a maximum percent induction $< 1.5\%$ total cells.

Dynamics of DNA damage induction in response to Carboplatin in Hair Follicles

Compared to DMSO treatment alone, carboplatin culture increased gH2AX labeling across all Donor 1 time-points from between a 1.9 fold increase at 24 hours to a 3.8 fold increase at 28 hours. However, two-tailed Student's T-tests applied to the data found that only the increase after 28 hours was significantly different compared to the time matched DMSO control group ($p=0.007$). The results are summarized in Appendix 1 as Figure 7.

The response to DNA damaging agent, carboplatin, was not significant in Donor 2. The percentage of cells positive for gH2AX was actually decreased in Donor 2 after treatment with carboplatin for 17 hours. Treatment with carboplatin for 24 and 28 hours resulted in an extremely small and non-significant fold increase in gH2AX. The results are summarized in Appendix 1 as Figure 7.

Labeling was significantly increased in Donor 3 with carboplatin treatment across all timepoints ranging from a 4.8 fold increase at 17 hours to a 12.5 fold increase at 24 hours compared to time matched DMSO treated controls. All changes in the percentage of cells that stained positive for gH2AX after treatment with carboplatin for 17, 24, and 28 hours were significant ($p=0.008$, 0.0009 , 0.0006 respectively). The results are summarized in Appendix 1 as Figure 7.

The non-robust and variable changes in gH2AX staining after treatment with carboplatin in Donors 1 and 2 followed by the significant induction in gH2AX staining at all timepoints in Donor 3 not only speaks to the high level of variability between hair follicles from a single donor, but the high variability in response between donors.

Evaluation of Carboplatin-induced DNA Damage in Hair Follicles by pChk1 at Multiple Timepoints

All compound treatments failed to induce any significant changes in labeling levels at any timepoint. There was a large amount of variability between hair follicles from the same donor. In addition, the number of positively labeled cells was low in all culture groups across all timepoints. Furthermore, the labeling that was determined as positive was so slight that it was difficult to detect any meaningful response when considering the variability between hair follicles. The results are summarized in Appendix 2 as Figure 8.

Data for Donor 3 at 17 and 24 hours was excluded due to the fact that the immunohistochemistry run was an outlier and produced slightly more intense nuclear staining when compared to other immunohistochemistry runs. To ascertain whether the data from this run should be included or excluded from statistical analyses, Dixon's Q test for outliers was completed on the treatment group means across all three donors and

found statistically that the affected samples should be removed (rejecting the null hypothesis that the outlier should be preserved, $p > 0.05$).

Effect of Ex Vivo Treatment on DNA Damage Induction in Hair Follicles

Ex vivo culture of the hair follicles was shown to affect gH2AX labeling. The data is summarized in Appendix 3 as Figure 9 and illustrates that maintenance in media alone was sufficient to affect gH2AX labeling. In hairs from donor 1, a decrease in total labeling, compared to freshly plucked, was observed when hairs were maintained ex vivo. Two-tailed Student's T-tests applied to the untreated culture (media alone) group found that the reduction in labeling from fresh was significant only after 28 hours of culture ($p = 0.007$). Comparison of the 0.1% DMSO vehicle with the media control group showed further reductions across all timepoints where only the reduction in labeling due to the addition of DMSO to the culture media at 17 hours was statistically significant ($p = 0.032$). Results are illustrated in Appendix 3 as Figure 9.

These findings were also seen in donor 3 where cultured hairs revealed a statistically significant reduction in labeling across all DMSO treated groups when compared to hairs cultured in media alone. The reduction in gH2AX labeling at the 17 and 28-hour timepoints were statistically significant ($p = 0.02, 0.023$, respectively). Differences seen between fresh hairs fixed immediately and the hairs cultured in media alone were not significant. Results are illustrated in Appendix 3 as Figure 9. Thus, the media and DMSO treatment may cause down-regulation of the damage signal, indicating

that we may be missing something in the ex vivo setting where we have to culture the hairs in DMSO.

In contrast to donors 1 and 3, there was an increase in gH2AX labeling when hairs were placed into culture media and a further increase in gH2AX labeling with the inclusion of DMSO alone into the media. Cultured and fresh hairs, however, from donor 2 displayed much lower levels of gH2AX labeling compared to donor 1 and 3. Analysis of the data proved all changes in total labeling as result of culture or DMSO to be insignificant. Results are illustrated in Appendix 3 as Figure 9.

In hairs from donors 2 and 3 a significant decrease in pChk1 labeling was observed compared to freshly plucked samples when hairs were maintained ex vivo (untreated) across all culture timepoints. However, though significant, this statistically significant result only constitutes a very small reduction from 2.1% to 1.48% to below 1% across all timepoints for donors 2 and 3 respectively. Additionally, the number of positively labeled cells was low in all culture groups. Results are illustrated in Appendix 4 as Figure 10.

In the analysis of both gH2AX and pChk1, expression of DNA damage markers at baseline (fresh, fixed immediately) was variable. Similarly, follicle-to-follicle variability within a treatment group was high. This makes determining a response to stimuli (ie. ex vivo treatment with carboplatin) difficult.

DNA Damage Induction in Whole Blood as Measured by γ H2AX and pKAP1 Double Positive Cells

In examining the induction in DNA damage in whole blood, results indicate that the ex vivo stimulation with DNA damaging agents lead to minimal enhancement of DNA damage. Specifically, after ex vivo stimulation with carboplatin, etoposide, and irinotecan there was minimal non-significant induction in DNA damage markers pKAP1 and γ H2AX using any of the DNA damaging agents. The human whole blood was treated with 2 Gy of radiation as a positive control as it has been shown to successfully induce damage ex vivo. Results from the samples treated with radiation indicate that the staining and evaluation via flow cytometry was successful. Maximum DNA damage was seen 1 hour post radiation, and was almost 100% resolved by 8 hours post treatment. Results are illustrated in Figure 5 below.

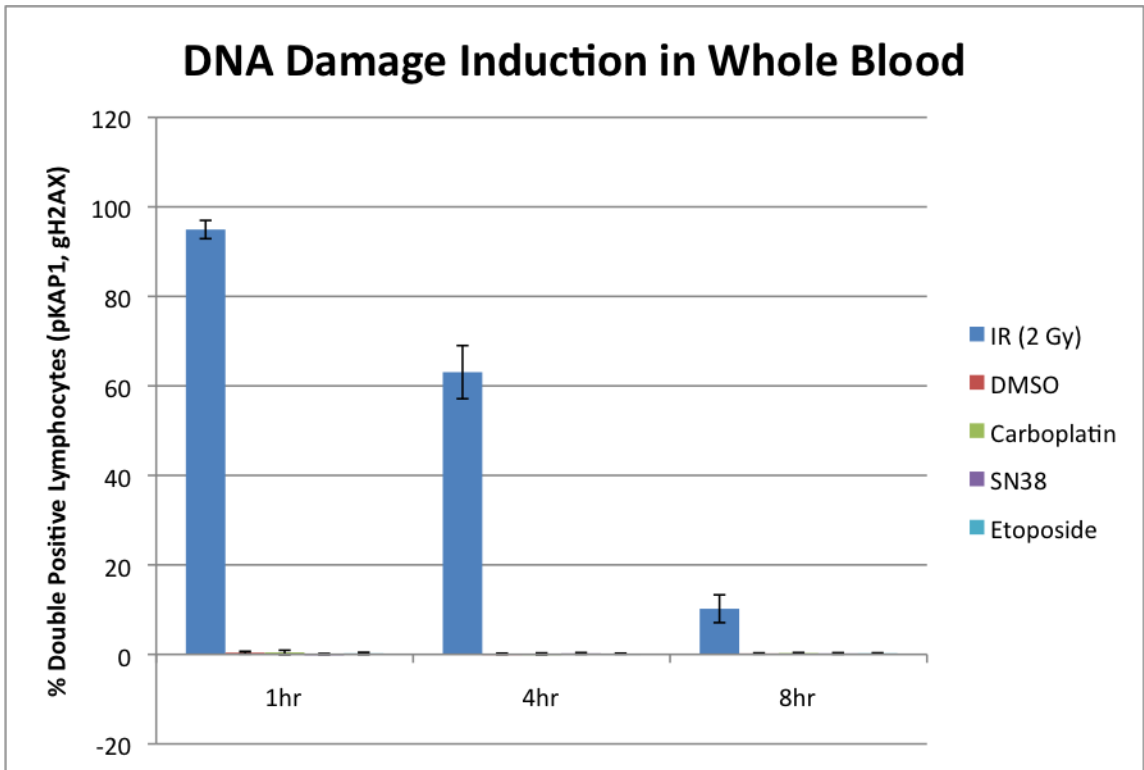


Figure 5. Ex vivo induction in DNA damage by DNA damaging agents not successful in whole blood as measured by damage markers, pKAP1 and gH2AX. Radiation successfully induced DNA damage with maximum damage seen at 1-hour post treatment.

Individual data points for each of the DNA and positive control listed below with standard deviations generated by pooling the duplicate replicates and three individual donors.

Table 4. Percent double positive lymphocytes for pKAP1, gH2AX (standard deviation).

	IR (2 Gy)	DMSO	Carboplatin	SN38	Etoposide
1 hour	94.9 (2.04)	0.04 (0.29)	0.46 (0.48)	0.10 (0.09)	0.24 (0.22)
4 hour	63.1 (5.94)	0.15 (0.12)	0.15 (0.18)	0.24 (0.16)	0.18 (0.09)
8 hour	10.2 (3.11)	0.22 (0.11)	0.30 (0.11)	0.23 (0.14)	0.25 (0.10)

Results indicate that ex vivo induction in DNA damage using chemotherapeutic agents was unsuccessful at all timepoints as measured by damage markers, pKAP1 and gH2AX. However, ex vivo treatment of whole blood with 2 Gy radiation successfully induced damage at all timepoints with maximum damage observed 1 hour after treatment.

In vivo DNA Damage Response after Treatment with Etoposide in Rats

After the ex vivo treatment with DNA damaging agents in human whole blood was shown to be unsuccessful, DNA damage response was explored in vivo. Specifically, rats were treated with two doses of etoposide and blood was collected at 1, 2, 7 and 24 hours post treatment. Data from individual animals in each of the treatment groups (n=4) was pooled. Results indicate that DNA damage was successfully induced and a measurable increase compared to DMSO was detected at 1 and 2 hours post dose in the 10mg/kg dose and at all timepoints in the 15mg/kg dose. However, increases 1 hour post

the 10mg/kg dose ($p=0.019$) and at 1 and 2 hours post the 15mg/kg dose ($p=0.004$ and 0.022 , respectively) were the only statistically significant results. Full results are illustrated in Figure 6 below.

The *in vivo* DNA damage induction occurred in a dose-dependent fashion in rats treated with etoposide. Specifically, the 15mg/kg dose induced a higher level of DNA damage as measured by lymphocytes positive for both pKAP1 and γ H2AX than the 10mg/kg dose at all timepoints. However the only timepoint that showed a statistically significant difference between the two doses was the 1-hour timepoint ($p=0.0007$). Results are illustrated in Figure 6 below. The ability to induce a DNA damage response *in vivo* in rats highlights the difference between the *ex vivo* and *in vivo* etoposide treatments and indicates that blood may be a viable surrogate to tumor tissue when monitoring DNA damage biomarkers.

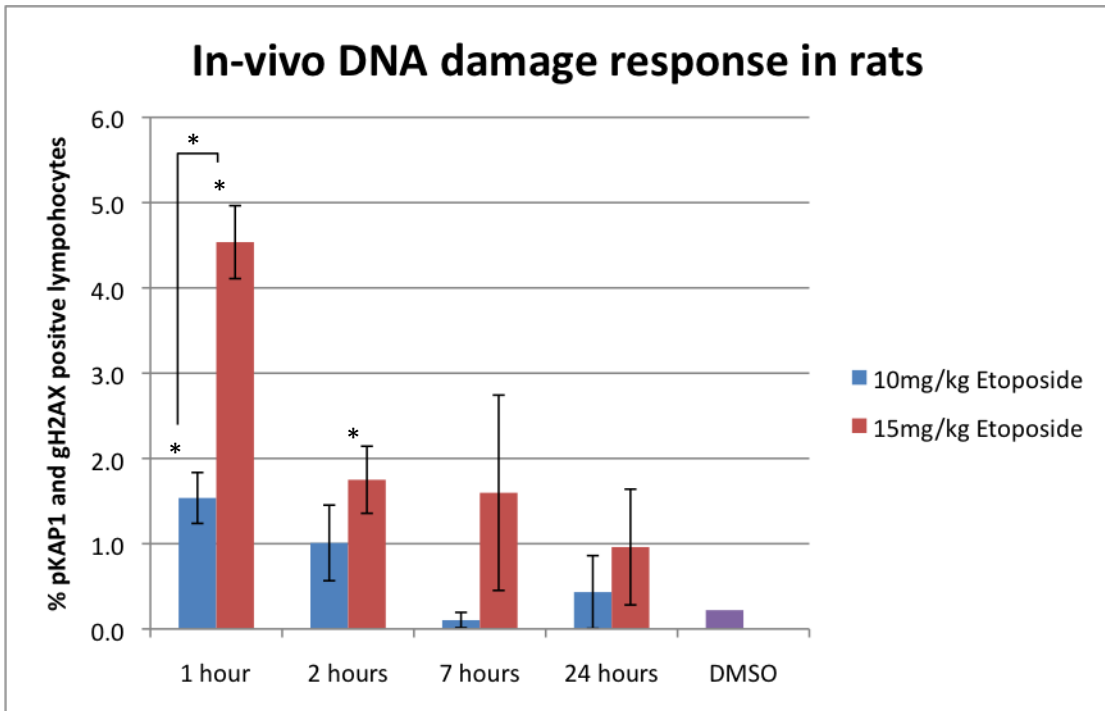


Figure 6. Treatment with etoposide significantly induces DNA damage response in whole blood in rats compared to DMSO control. *indicates statistical significance $p < 0.05$ using Student's t-test.

A group of animals (n=4) was also treated with 2 Gy of radiation as a positive control. Animals treated with 2 Gy radiation had 99.0% of lymphocytes positive for gH2AX and pKAP1 at 1 hour (data not shown).

Chapter IV

Discussion

Our results indicate that both hair follicles and whole blood may be suitable tissue types for biomarker monitoring of the mechanistic effects of DNA damaging agents in oncology trials. With that in mind, we observed quite a bit of variability in both tissue types in ex vivo specimens and less variability in vivo. These data indicate that optimization and validation of specific biomarkers after treatment with chemotherapeutic agents may need to be done using hair and blood from in vivo experiments given the potential limitations of ex vivo material, especially in blood. With proper in vivo validation, assessment of on-mechanism biomarkers in blood and hair may be used in dose and schedule selection in future generations of DNA damaging agents.

Biomarker Monitoring in Hair Follicles

This thesis has confirmed that hair follicles can be used for on mechanism biomarker monitoring in response to DNA damaging agents. Hair follicles can be used for biomarker monitoring in response to DNA damaging agents. We observed that up to 70% of cells stained positive for gH2AX after treatment with the DNA damaging agent

etoposide. However, the DNA damage induction seen by measuring pChk1 staining after treatment with DNA damaging agents was markedly less when compared to gH2AX staining. The maximum induction in DNA damage was less than 2% after treatment with Irinotecan (SN38) when using pChk1 as a marker of DNA damage. This could be due to the fact that H2AX is not only phosphorylated at Ser139 in response to DNA damaging agents, gH2AX can also be generated during DNA replication, as a consequence of apoptosis or in response to residual DNA damage. H2AX is recruited directly to the damaged DNA and is phosphorylated to activate the DNA damage response (DDR) by multiple DDR pathways including homologous recombination (HR) and base excision repair (BER) pathways. Conversely, Chk1 is activated mainly by ataxia telangiectasia and Rad3-related protein (ATR) through phosphorylation after ATR is activated in response to DNA damage. Thus, as pChk1 is slightly downstream of the DNA damage response, lessening its expression in response to DNA damage compared to H2AX, it explains some of the differences between the degree of activation of pChk1 and the degree of activation of H2AX.

Additionally, phosphorylation of Chk1 may be more transient when compared to H2AX and is specific to the S-phase of the cell cycle where as the activation of H2AX can occur in all phases of the cell cycle. Thus, the DNA damage induction could be very strong, but the timepoints in which we were monitoring pChk1 may not have captured the maximum induction. Future studies could explore a more granular time course to pinpoint maximum induction of pChk1 in response to DNA damaging agents.

DNA Damage Induction in Hair is Variable by Agent and Donor

Our results indicate that measurement of gH2AX can be used as an on mechanism biomarker of DNA damage to carboplatin, etoposide, and irinotecan. Although the three DNA damaging agents tested did not induce the same levels of DNA damage, damage induction was detectable in response to all agents tested. Across all three donors, etoposide induced the greatest DNA damage as measured by gH2AX. Treatment with etoposide had about a two-fold greater induction in DNA damage compared to carboplatin and irinotecan in the same donors. This data suggests that treatment of surrogate tissues with a topoisomerase II inhibitor that causes direct double strand breaks may be a more potent inducer of damage response in surrogate tissues.

The variability in damage between agents might further be attributed to the concentrations used during culture. For each of the three agents, the IC90 concentration in cancer cell lines was used. Perhaps the IC90 concentrations in cell lines did not translate exactly to hair follicles. The only way to know if the concentrations of carboplatin and irinotecan were low is to do a full dose titration to understand the dose/response relationship.

Treatment with carboplatin also resulted in the induction in DNA damage in hair follicle. Here we saw a significant increase in the DNA damage marker, gH2AX, in one donor after 24 hours of ex vivo treatment with carboplatin. As a result, we decided to explore multiple timepoints to understand if treatment for 24 hours resulted in the

maximum response or if we were missing the window of highest induction. Hairs were treated, ex vivo, with carboplatin for 17, 24, and 28 hours. Results indicated that 28 hours was the timepoint in which we saw the most robust and consistent induction across all three donors. Thus, while it seems from this experiment that 28 hours is best, additional work to evaluate other timepoints would be useful before using these assays in the clinic.

Finally, treatment with irinotecan (active agent, SN38), a topoisomerase I inhibitor, did not result in a statistically significant inductions in DNA damage response as measured by gH2AX or pChk1. Topoisomerase I is responsible for relaxation of DNA supercoils by making single and double stranded cuts in the DNA, crossing the stands through one another then, resealing the breaks. Inhibition of topoisomerase I leads to the inhibition of DNA replication and transcription. Perhaps the difference in MOA explains the differences in the amount of damage observed.

There was significant variability between donors as well. One of the donors did not show a significant induction in gH2AX at any of the timepoints. Furthermore, the level of induction in one of the donors that showed a statistically significant induction in gH2AX at 28 hours was almost 40% higher than the other donor that the other statistically significant increase at 28 hours.

Effect of Ex Vivo Culture on Hair Follicles

In this thesis, DNA damage response was evaluated in an ex vivo environment. However, in the clinic, patients would typically be treated intravenously with DNA damaging agents, and the hair follicles would be exposed to the agents in vivo due the vascularization of the scalp. To more fully understand the utility of hair follicles as a surrogate tissue to assess DNA damage response, in vivo studies in patients or rodents should be performed. However, in our experiments, the hair follicles were plucked from healthy volunteers, placed in an ex vivo culture containing cell culture media supplemented with the DNA damaging agent. Hair follicles collected in the clinic would not be exposed to DMSO and the body would be metabolizing the DNA agent such that the concentration of the agent would not be constant. In addition, the body would maintain the hair follicles and vascularization of the scalp would keep the epithelial cells alive. Thus, we examined the effect the ex vivo culture itself had on the hair follicles.

We observed that maintenance in media alone was sufficient to induce gH2AX labeling. Thus, there was some level of cell death occurring just because the hair follicles were being maintained outside of the body. Whether the increase is due to donor responses to plucking trauma, in intrinsic donor-specific characteristic, or as a result of prolonged culture is unknown. However, when comparing the level of DNA damage in vehicle treated hair follicles compared to untreated follicles in media alone, the hairs treated with DMSO showed increased damage compared to media. Thus, the addition of

vehicle alone was enough to elicit a response in DNA damage compared to the culture itself.

These effects due to the ex vivo culture were variable by donor. In donor 1, untreated hair follicles maintained in media and DMSO treated follicles resulted in decreased gH2AX when compared to hair follicles plucked and fixed immediately. In comparison, donor 2 showed an increase in gH2AX in hair follicles maintained ex vivo (untreated and DMSO treated).

Lastly, though we saw a statistically significant reduction in pChk1 in untreated hair follicles, the number of cells that were positive for pChk1 to begin with, in the freshly plucked hair follicles, was extremely low. In the two donors that demonstrated a statistically significant reduction, the number of cells positive for pChk1 was reduced from about 1.5 to 2% of cells to only about 0.5% in both donors. Thus, even though the results are statistically significant, the percent reductions are extremely low and the significance is not replicated in the DMSO treated group.

Biomarker Monitoring in Blood

Here we demonstrate minimal DNA damage induction in ex vivo treated blood and a robust induction in vivo after treatment with etoposide. Thus, results from whole blood experiments demonstrate discordance between ex vivo treated whole blood and whole blood taken and processed from mouse experiments. Given the discordance

between experiments and the fact that the ex vivo experiments failed to definitively prove that whole blood can be used as a surrogate tissue type for biomarker monitoring in the clinic, additional in vivo experiments are suggested prior to clinical use. Human whole blood from three healthy donors was treated with carboplatin, etoposide, and irinotecan for 1, 4, and 8 hours. Blood was also collected and treated with 2 Gy radiation and fixed after 1, 4, and 8 hours as a positive control. Channels on the flow cytometer were gated to identify cells that were double positive for γ H2AX and pKAP1. Samples treated with the various DNA damaging agents showed no increase in DNA damage response (DDR) markers compared to DMSO. Therefore, human whole blood treated ex vivo with three DNA damaging agents failed to induce a DNA damage response (DDR) as measured by DDR biomarkers, γ H2AX and pKAP1. This could be due, in part, to the fact that the blood was removed from the in vivo environment and is not undergoing DNA replication. Since these three DNA damaging agents work to inhibit DNA replication and transcription, treating whole blood in an ex vivo manner where the cells are not cycling would not result in a robust response in DDR markers. In addition, there is a significant gap between the time in which the blood is removed from the volunteer and the time it is treated with the DNA damaging agent. During this time, the blood is sitting at the bench at room temperature, which may affect the integrity of the blood components, adversely effecting the results.

In order to understand the effect ex vivo stimulation had on the results, the experiment was repeated in vivo in rats. Rats were treated with intraperitoneal (ip) etoposide or 2 Gy IR as a positive control. In vivo treatment with etoposide resulted in a

dose-dependent DDR where the higher dose of etoposide resulted in maximum damage at 1 hour post treatment. Specifically, treatment with etoposide resulted in about a 4-fold induction in DDR markers, γ H2AX and pKAP1, compared to DMSO. By the 24-hour timepoint, the compound had been fully metabolized by the rats, thus resulting in the lowest DDR. A key difference between the *in vivo* and *ex vivo* experiments is that compound is maintained at a constant concentration in the *ex vivo* experiment. In contrast, *in vivo* experiments more closely mimic human conditions where drug is metabolized over time allowing one to understand the relationship between drug exposure and biomarker response.

Taken together, these results indicate that DNA damage response can be monitored in whole blood. However, optimization and validation in an *ex vivo* setting may not be possible using chemotherapeutics that target DNA replication since whole blood is not undergoing DNA replication *ex vivo*. Radiation, however, can be used in *ex vivo* experiments, as it does not rely on the cell cycle to induce DNA damage. Specifically, radiation causes both single and double strand breaks in the DNA leading to genomic instability. Thus, radiation can be modeled in *ex vivo* experiments for clinical readiness where targeted agents may be combined with radiation for example.

Implications and Future Experiments

With a recent emphasis on molecularly targeted therapies oftentimes in combination with chemotherapeutic agents, biomarkers that are indicative of a drug's pharmacodynamic mechanism of action or illustrative of target engagement are essential. Biomarker monitoring in the target tumor tissue is not always possible, lending itself to the importance of finding surrogate tissues. Confirmation that both hair follicles and blood can be used to monitor “on-mechanism” changes induced by DNA damaging agents in a clinical oncology trial has large implications for the development of novel biomarkers. If surrogate tissues can be utilized in the clinic instead of tumor tissue biopsies, this will allow for the collection of more data at more timepoints with a decrease in patient burden.

One limitation of this thesis is the level of DNA damage induction in hair follicles seen with the chemotherapeutics tested. Examination of hair follicles *in vivo* in rodents or in humans may result in a better fold induction because the hairs are being maintained by the animal's vascular system instead of by cell culture media *ex vivo*. In addition, exploring more concentrations and timepoints to try to identify maximum induction may help to reduce variability. Additionally, other surrogate tissues could be explored in addition to hair follicles and blood. Skin, for example, could be a viable option for biomarker monitoring that is easily accessible and amenable to repeat sampling when compared to tumor tissue biopsies.

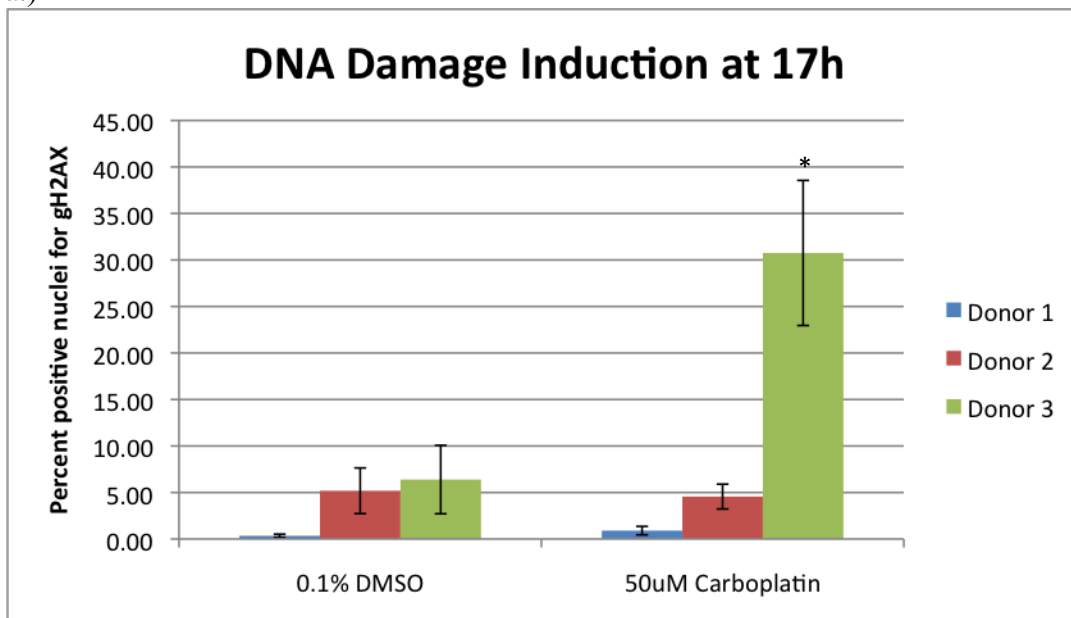
In conclusion, both hair follicles and whole blood are viable options for biomarker monitoring in the clinic and may serve as an alternative to a very burdensome procedure, the standard tumor tissue biopsy.

Chapter V

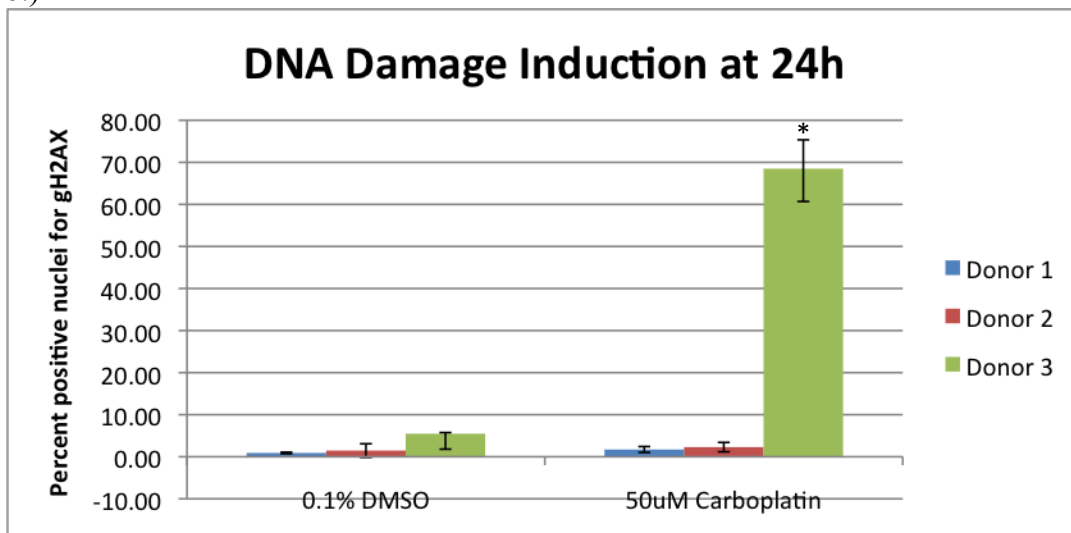
Appendices

Appendix 1. Treatment with Carboplatin Induced DNA Damage as Measured by gH2AX

a.)



b.)



c.)

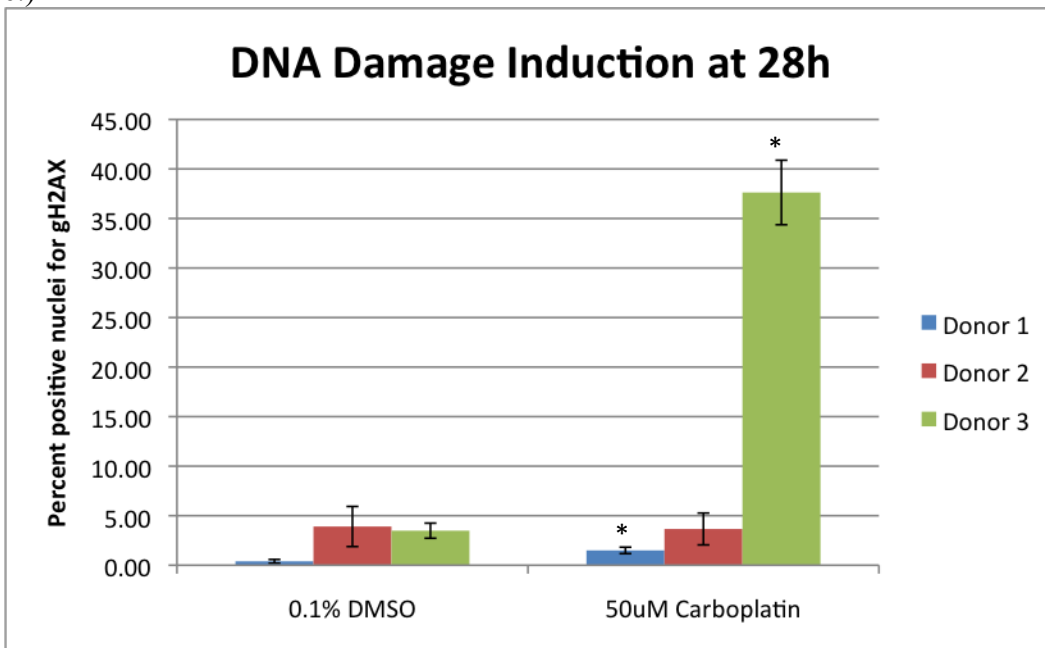
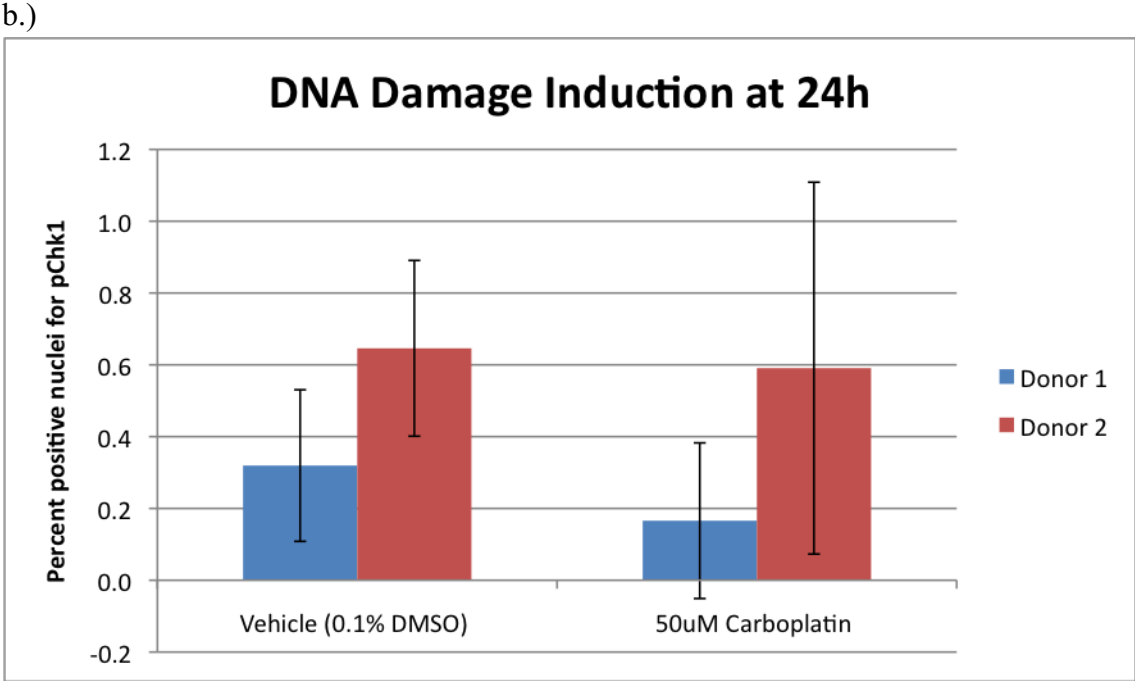
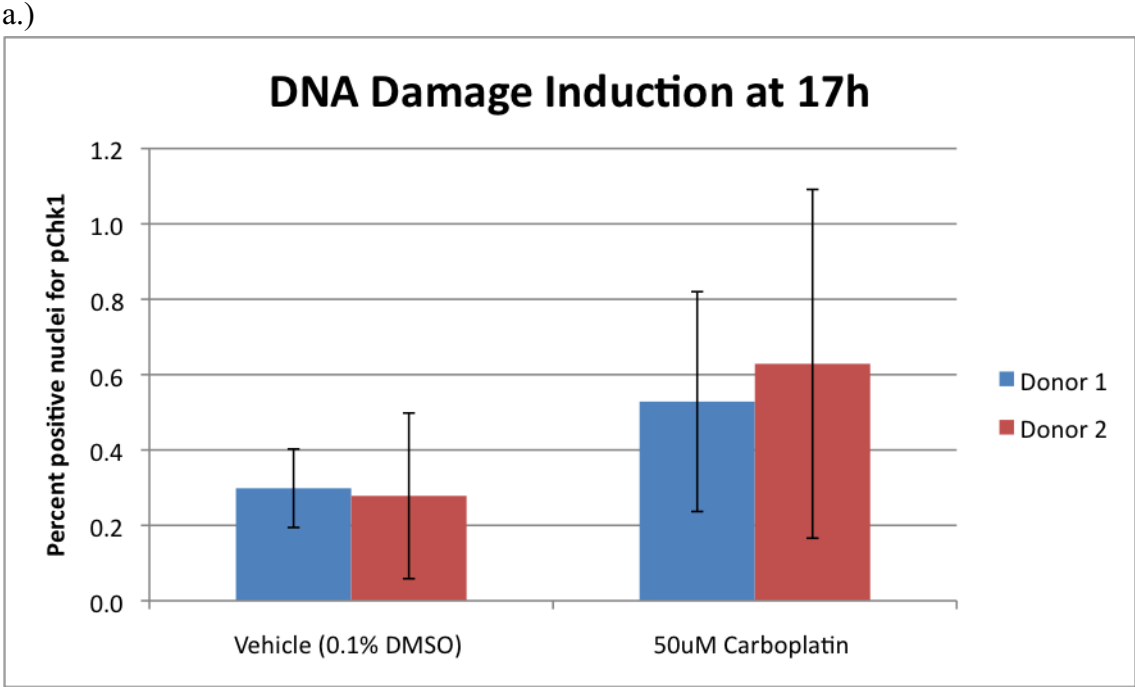


Figure 7. Treatment with Carboplatin induced DNA damage as measured by gH2AX. Percentage of Carboplatin-treated hair follicle cells positively stained for gH2AX, relative to DMSO treated controls. DNA damage induction at 17h (a), 24h (b), and 28h (c) after treatment with 50uM Carboplatin. * denotes significance (two tailed T-test against equivalent donor vehicle; $p < 0.05$)

Appendix 2. Treatment with Carboplatin Induced DNA Damage as Measured by pChk1



c.)

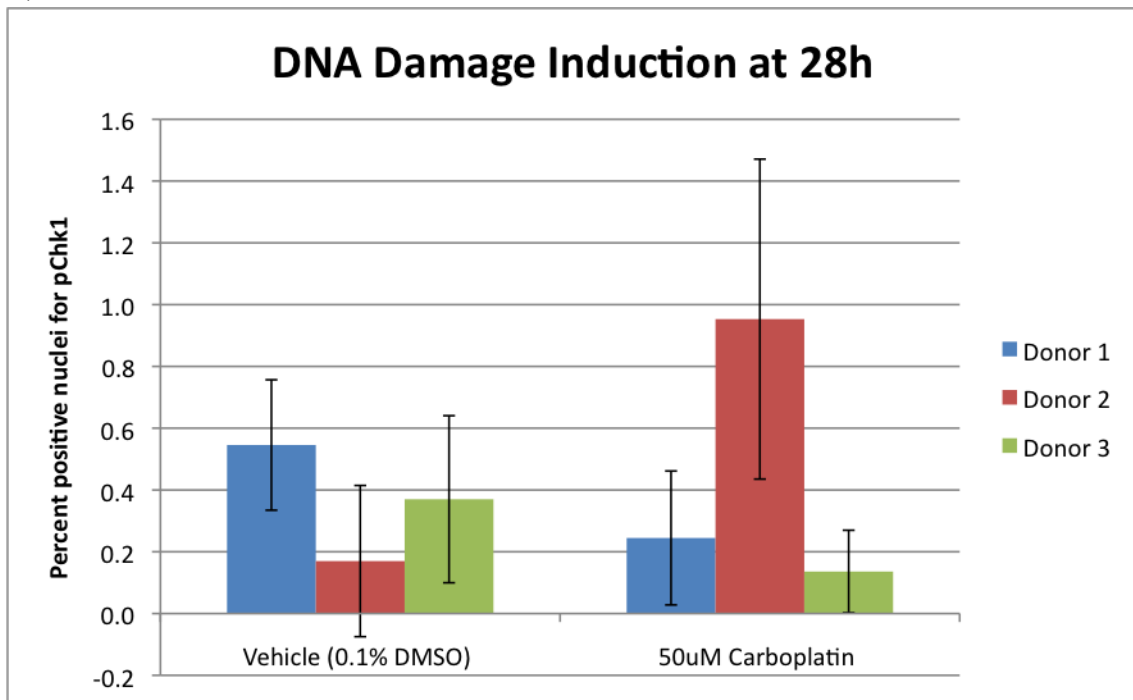
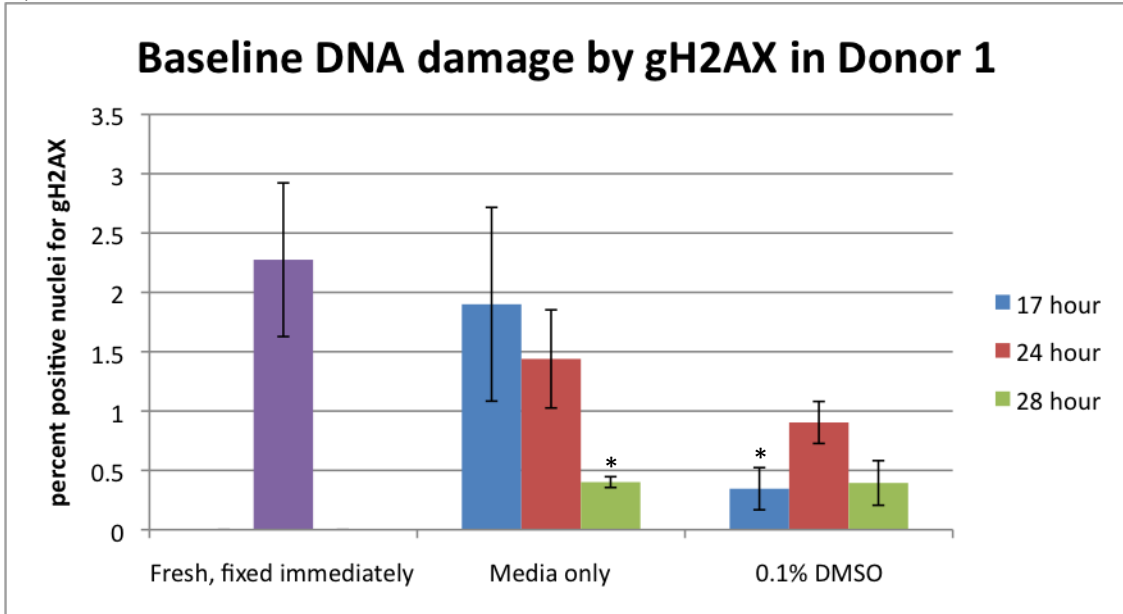


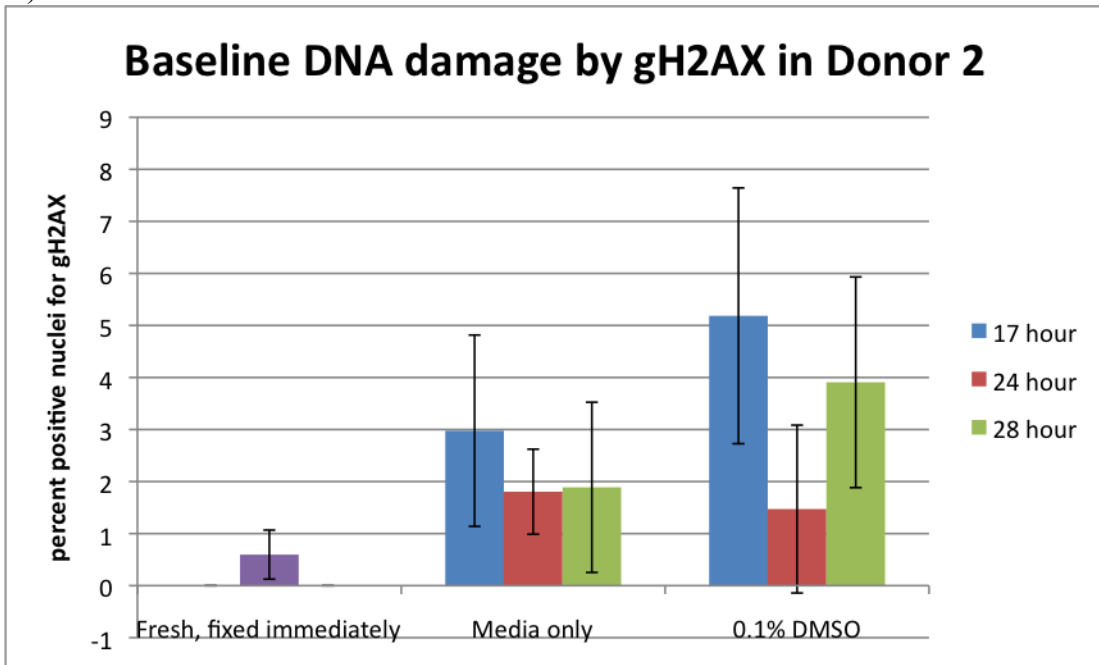
Figure 8. Treatment with Carboplatin induced DNA damage as measured by pChk1. Percentage of Carboplatin-treated hair follicle cells positively stained for pChk1, relative to DMSO treated controls. DNA damage induction at 17h (a), 24h (b), and 28h (c) after treatment with 50uM Carboplatin. * denotes significance (two tailed T-test against equivalent donor vehicle; $p < 0.05$)

Appendix 3. Baseline DNA Damage During Ex Vivo Culture as Measured by gH2AX

a.)



b.)



c.)

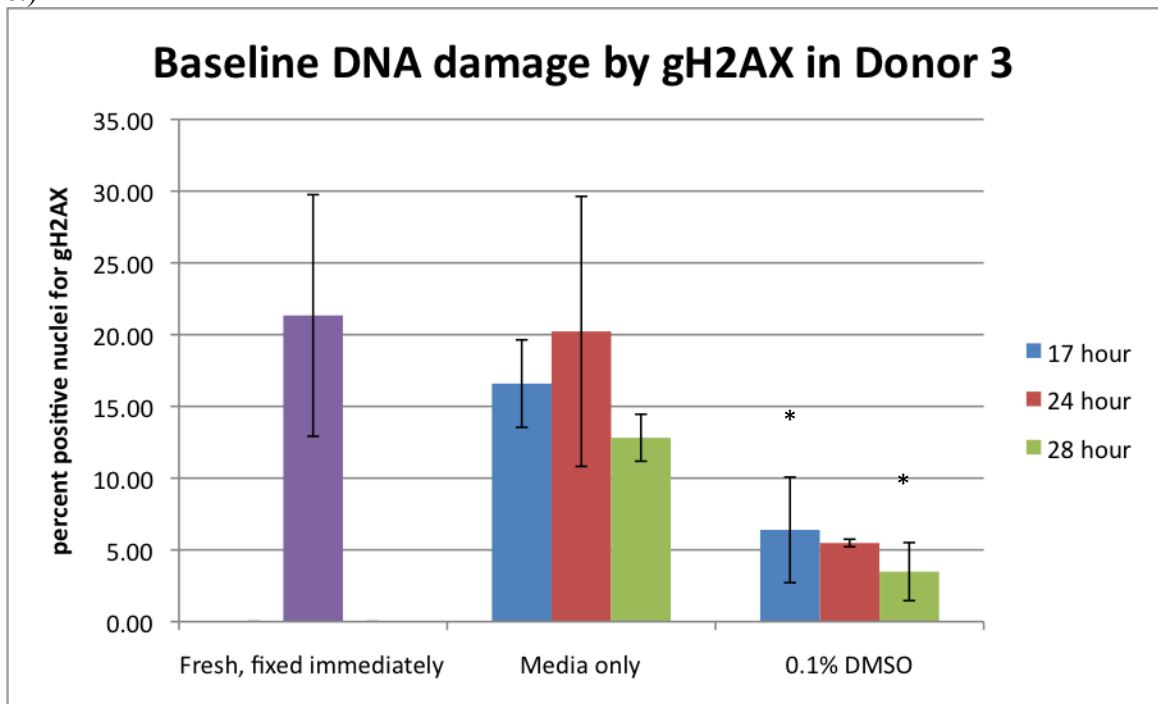
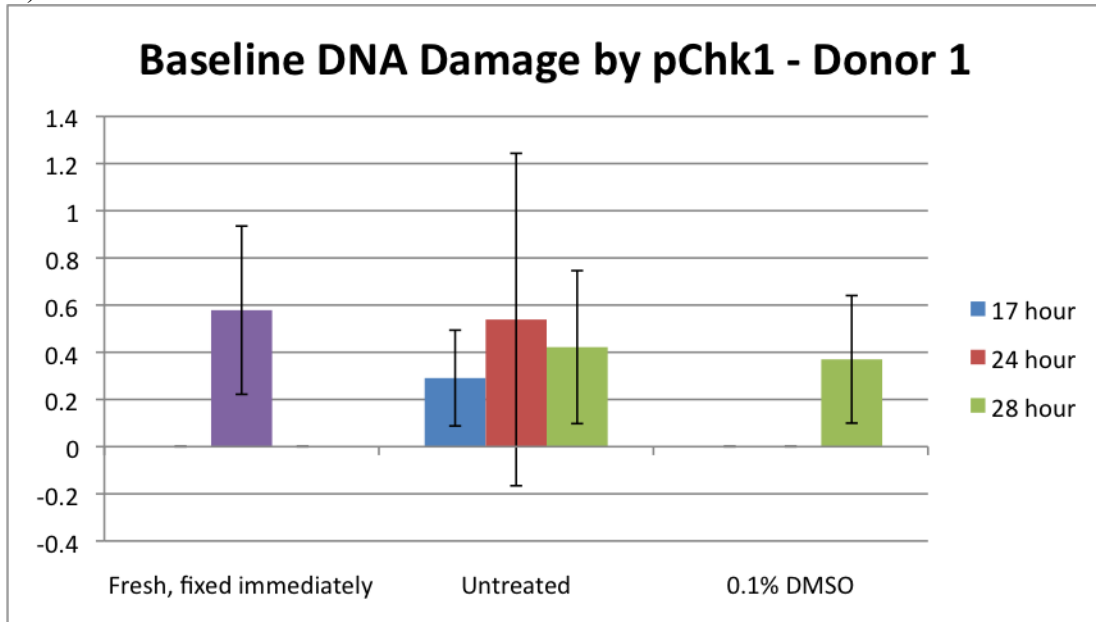


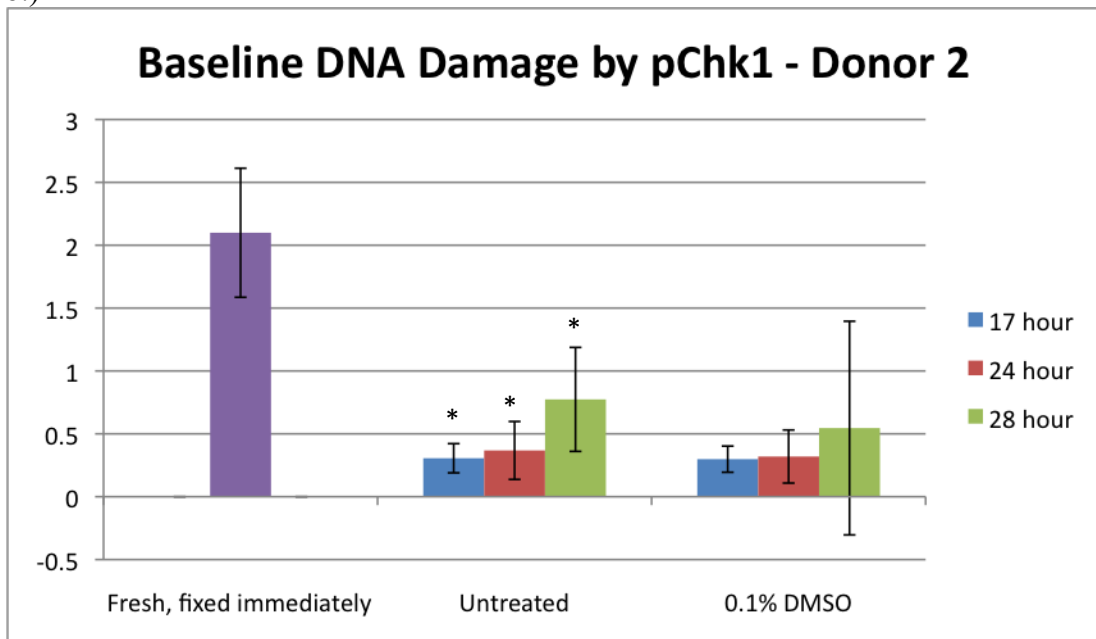
Figure 9. Baseline DNA damage during ex vivo culture as measured by gH2AX. DNA damage induction in donor 1 (a), donor 2 (b), and donor 3 (c) after culture with either media alone or media supplemented with 0.1% DMSO. * denotes significance (two tailed T-test equivalent against fresh hairs fixed immediately (media alone group) or media alone group (0.1% DMSO group)).

Appendix 4. Baseline DNA damage during Ex Vivo Culture as Measured by pChk1

a.)



b.)



c.)

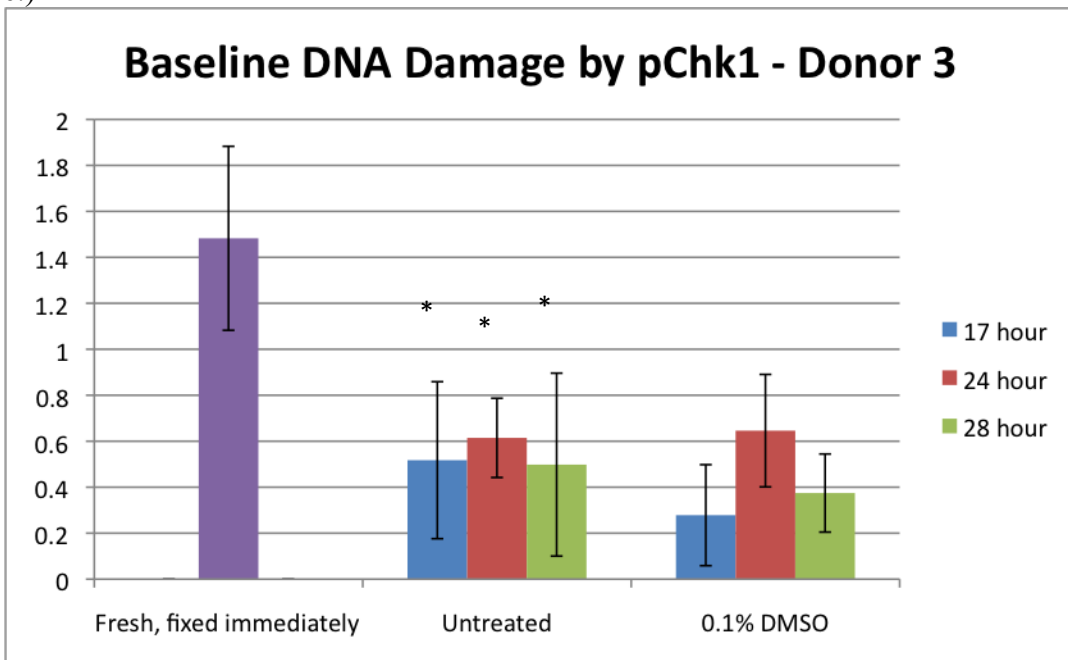


Figure 10. Baseline DNA damage during ex vivo culture as measured by pChk1. DNA damage induction in donor 1 (a), donor 2 (b), and donor 3 (c) after culture with either media alone or media supplemented with 0.1% DMSO. * denotes significance (two tailed T-test equivalent against fresh hairs fixed immediately (media alone group) or media alone group (0.1% DMSO group)).

References

- Alli, E., Sharma, V. B., Sunderesakumar, P., et al. (2009). Defective Repair of Oxidative DNA Damage in Triple-Negative Breast Cancer Confers Sensitivity to Inhibition of Poly(ADP-Ribose) Polymerase. *Cancer Research*, 69(8), 3589- 3596.
- Ang, J. E., Kaye, S., Banerji, U. (2012). Tissue-Based Approaches to Study Pharmacodynamic Endpoints in Early Phase Oncology Clinical Trials. *Current Drug Targets*, 13(12), 1525-1534.
- American Cancer Society. *Cancer Facts & Figures 2014*. Atlanta: American Cancer Society; 2014
- Camidge, D. R., Randall, K. R., Foster, J. R., Sadler, C. J., Wright, J. A., Soames, A. R., Laud, P. J., Smith, P. D., Hughes, A. M. (2005). Plucked human hair as a tissue in which to assess pharmacodynamic end points during drug development studies. *British J of Cancer*, 92(10), 1837-1841.
- DeVita, V., Chu, E. (2008). A History of Cancer Chemotherapy. *Cancer Research*, 68(21),8643-8653.
- Gho, C. G., Braun, J. E. F., Tilli, C. M. L. J., Neumann, H. A. M. (2004). Human follicular stem cells: their presence in plucked hair and follicular cell culture. *British J of Derm*, 150, 860-868.

- Gramont, A., Watson, S., Ellis, L. M., Rodon, J., Taberero, J., Gramont, A., Hamilton, | S. R. (2014). Pragmatic issues in biomarker evaluation for targeted therapies in cancer. *Nature Reviews Clinical Oncology*. Published online 25 November 2014. <http://dx.doi.org/10.1038/nrclinonc.2014.202>
- Hanahan, D., Weinberg, R. A., The Hallmarks of Cancer. *Cell*, 100(1), 57-70.
- Hosoya, N., Miyagawa, K. (2014). Targeting DNA Damage response in cancer therapy. *Cancer Science*, 105(4), 370-388
- Ji, J., Kinders, R. J., Zhang, Y., Rubinstein, L., Kummar, S., Parchment, R. E., Tomaszewski, J. E., Doroshov, J. H. (2011). Modeling Pharmacodynamic Response to the Poly(ADP-Ribose) Polymerase Inhibitor ABT-888 in Human Peripheral Blood Mononuclear Cells. *PloS ONE*, 6 (10), 1-8.
- Lee, J., Hays, J., Noonan A., Squires, J., Minasian, L., Annuziata, C., Wood, B. J., Yu, M., Calvo, K. R., Houston, N., Aza, N., Kohn, E. (2013). Feasibility and safety of sequential research-related tumor core biopsies in clinical trials. *Cancer*, 119(7): 1357-1364.
- Loughran, C. F., Keeling, C. R. (2011). Seeding of tumour cells following breast biopsy: a literature review. *The British Journal of Radiology*, 84:869-874.
- Ma, B. B. Y., Chan, A. T. C. (2006). Transcriptional profiling of tumor biopsies in oncology trials – a ‘window’ of opportunity for evaluating new drugs in nasopharyngeal cancer? *Annals of Oncology*, 17(11): 1611-1613.
- O’Reilly, T., McSheehy, P. (2010). Biomarker development for the Clinical Activity of the mTOR inhibitor Everolimus (RAD001): Processes, Limitations, and Further Proposals. *Translational Oncology*, 3(2): 65-79.

- Overman, M. J., Modak, J., Kpetz, S., Murthy, R., Yao, J. C., Hicks, M. E., Abbruzzese, J. L., Tam, A. L. (2013). Use of Research Biopsies in Clinical Trials: Are Risks and Benefits Adequately Discussed? *J of Clin Oncology*, 31(1): 17-22.
- Paweletz, C. P., Andersen, J. N., Pollock, R., Nagashima, K., Hayashi, M. L., Yu, S. U., Guo, H., Bobkova, E. V., Xu, Z., Northrup, A., Blume-Jensen, P., Hendrickson, R. C., Chi, A. (2011). Identification of Direct Target Engagement Biomarkers for Kinase-Targeted Therapeutics. *PLoS ONE*, 6(10), e26459.
- Randall, K., Foster, J. R. (2007). The Demonstration of Immunohistochemical Biomarkers in Methyl Methacrylate-Embedded Plucked Human Hair follicles. *Toxicologic Pathology*, 35:952-957.
- Rojo, R., Tabernero, J., Albanell, J., Cutsem, E. V., Ohtsu, A., Doi, T., Koizumi, W., Shirao, K., Takiuchi, H., Cajal, S. R., Baselga, J. (2006). Pharmacodynamic Studies of Gefitinib in Tumor Biopsy Specimens From Patients With Advanced Gastric Carcinoma. *J of Clin Oncol*, 24 (26): 4309-5620.
- Sawyers, C. L. (2008). The Cancer Biomarker Problem. *Nature*, 452(3): 548-552.
- Shiotani, B., Zou, L. (2009). ATR signaling at a glance. *J of Cell Science*, 122(3), 301-304.
- Takimoto, C. H. (2009). Pharmacokinetics and pharmacodynamic biomarkers in early oncology drug development. *Eur J Cancer*, 45(Suppl 1): 436-438.
- Williams, R., Baker, A., Ihle, N., Winkler, A. R., Kirkpatrick, L. (2006). The skin and hair as surrogate tissues for measuring the target effect of inhibitors of phosphoinositide-3-kinase signaling. *Cancer Chemother Pharmacol*, 58(4): 444-450.

Yap, T. A., Walton, M. I., Hunter, L. K., Valenti, M., Brandon, A., Eve, P. D., Ruddle, R., Heaton, S. P., Henley, A., Pickard, L., Vijayaraghavan, G., Caldwell, J. J., Thompson, N. T., Aherne, W., Raynaud, F. I., Eccles, S. A., Workman, P., Collins, I., Garrett, M. D. (2010). Preclinical Pharmacology, Antitumor Activity, and Development of Pharmacodynamic Markers for the Novel, Potent AKT Inhibitor CCT128930. *Mol Cancer Ther*, 10(2): 360-371.