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Polymerase Eta

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Development of a High-throughput Screening Assay for Potential Inhibitors Against DNA Polymerase Eta

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Abstract

All living cells perform DNA replication through a group of enzymes called DNA polymerases. Unfortunately, DNA is constantly exposed to external and internal agents capable of causing damage. DNA damage causes blocks to replication that can be overcome through a special class of DNA polymerases, called the Y-family polymerases; which are able to traverse DNA lesions. Cisplatin is a common chemotherapeutic agent that damages DNA and has been used to treat cancer. However, many patients become resistant to cisplatin. The chemoresistance to cisplatin treatment is due in part to damage bypass performed by the specific Y-family polymerase, polymerase eta. The potential role of Y-family DNA polymerases in the pathway of chemotherapeutic resistance offers a novel target for inhibition.

To cultivate this thesis, I will provide a brief introduction of cancer and how DNA plays a role in the development of the disease. I will then discuss the various types of DNA damage and how they affect the replication process resulting in mutagenesis. Following that, I will begin discussing several types of repair processes that our cells have adopted to tolerate DNA damage. This will lead me to hone in one specific mechanism called translesion synthesis, and discuss how through the recruitment of a special class of DNA polymerases called the Y family polymerases allows for DNA replication to continue without fixing the damage, and how this plays a role in chemotherapeutic resistance. From this I will get into the specifics of my project, centered on DNA polymerase eta's role in cisplatin resistance which led to implementation of a high-throughput screening assay to identify potential small molecule targets against Y-family polymerase eta. I will conclude by summarizing the overall significance of the work done, and discuss research work that is currently being done on the inhibition of Y-family polymerases, as well as conclude with what future work can be done regarding this project.

Introduction

Since the beginning of humanity, cancer has plagued life in all forms.[1] Ancient civilization art and writings have reported cancer in South America, Asia and Egypt. Presently, the disease affects all species of higher animals.[1] In the United States, cancer is one of the leading causes of human death with approximately 1500 deaths a day.[2] In ancient civilizations, malignant transformations were likely attributed to the work of the gods. It was not until the Middle Ages, it became apparent that cancer was either inherited or a result of environmental exposure.[1] Today, it is commonly accepted that many cancers are not inherited and that tumor formation is a result of cumulative exposure to a multitude of environmental agents.[1] Epidemiological studies have supported this idea and show incidences of cancer related to geography and environment, rather than genetic influences.[1] Associations between environmental agents and incidences of cancer are very strong. One example is exposure to UV-radiation given off by the sun.[1] Exposure to it results in the formation of bulky thymine-dimer lesions that interfere with the replication process of DNA.[1]

Cancer can be the result of the production of a cell that is no longer restrained by normal growth control mechanisms.[3] Chemicals that can effect these cellular changes to yield cancer or malignant cells are called carcinogens.[3] One of the primary targets of chemicals in living organisms is the genetic material called DNA.[3] DNA is the hereditary material essential to all living cells and encodes for all of the organisms' essential functions.[4] Carcinogens alter the DNA of the target cell, causing either the replication of DNA to stop (cell death) or undergo mutations (mistakes) while copying the DNA base sequences.[4] When expressed, these error-containing DNA sequences may then produce cells with inappropriate responses to normal growth control restraints.[4]

It is now understood that modification of DNA is the initial step in the progression of tumor formation. The initiation stage involves DNA damage that alters the DNA's coding ability. If the

lesions are not repaired in an error-free manner, replication across these lesions can be highly mutagenic.[4] The resulting altered DNA sequence created by the replication across these lesions is further "fixed" by the replication machinery.[4] The altered sequence of DNA when expressed produces proteins and cells with altered function that progress into cancer.[4]

DNA Damage and Repair

Cancer cells do not possess the normal signals that regulate the cell cycle.[4] Unlike normal cells in culture, they do not stop dividing when growth factors are depleted. They are able to elude normal cell cycle regulation and divide unchecked resulting in the formation of a tumor.[4] Moreover, cancer cells can go on dividing indefinitely if sufficient nutrients are present, essentially making them immortal.[4] This is due in part to the cells being able to evade the normal controls that trigger a cell to undergo apoptosis when something goes wrong in the cell-cycle process such as an irreparable mistake during the DNA synthesis stage.[4,8] It is now understood that modification of DNA is the initial step in the progression of tumor formation.[4,8] The initiation stage involves DNA damage that alters the DNA's coding ability.[10] If the lesions are not repaired in an error-free manner, replication across these lesions can be highly mutagenic.[10] The resulting altered DNA sequence created by the replication across these lesions is further "fixed" by the replication machinery.[10] The altered sequence of DNA when expressed produces proteins and cells with altered function that progress into cancer.[4,10]

Before we can examine how DNA is mutated by carcinogenic damage, we must first understand the chemical structure of the molecule.[7,10] The genetic material of all life forms consists of a macromolecule called deoxyribonucleic acid (DNA).[8] DNA is a polymer, a large molecule that consists of many smaller molecules called monomers linked together. The monomers that make up DNA are called nitrogenous bases.[8] The four nitrogenous bases that make

up DNA are adenine, cytosine, guanine and thymine, denoted by the letters A, C, G and T.[8] The bases are held together via hydrogen bonds; intermolecular force that is strong enough to stably hold together DNA strands, yet easy to break.[8] Overall, the structure of DNA (Figure 1) resembles a double helix, with a sugar phosphate backbone on the outside of the helix and the nitrogenous bases orientated towards the central axis.[8] DNA carries the instructions for an organism to develop, survive, and reproduce.[8]

The replication of DNA is vital to a cell's survival. When DNA replication occurs, the strands of the DNA double helix separate, the hydrogen bond breaks at the central axis, (figure 1) and a new complementary strand of DNA is synthesized on each of the two parental template strands.[9] These newly synthesized strands of DNA are termed daughter strands, and are identical to the original strand.[9] DNA replication is an intricate process that is carried out in a series of controlled steps.[9] The first step to occur is the unwinding of the DNA double helix. This is done by an enzyme called helicase, whose purpose is to cleave the hydrogen bonds between the DNA base pairs.[9] Once the bonds are broken and the strands are separated, each strand serves as a template guide for the insertion of a complementary set of bases on the strand being synthesized (figure 1).[9] The new deoxynucleoside triphosphates (dNTP)

Original double stranded DNA

New strand   Original strand

Original strand   New strand

Figure 1. Structure of DNA double helix, and illustration of replication.

are added in a specific orientation. The incoming dNTP is covalently linked to the 3' carbon on the pentose as the second and third phosphates are removed together as a molecule of pyrophosphate.[9] The nucleotides are added in a way so that a purine always pairs with a pyrimidine and vice versa meaning that adenine is always inserted across a thymine and guanine is inserted
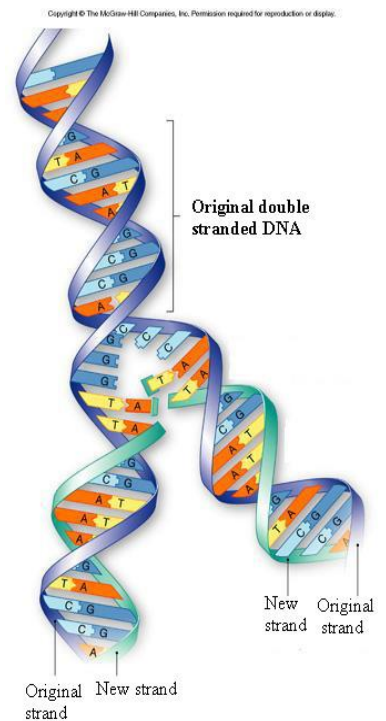
across from a cytosine.[9] This cellular process is carried out by a large biomolecule, called DNA polymerase.[9]

The DNA polymerase binds to one strand of the DNA and reads along it in a 3'$\rightarrow$5' fashion, adding new nucleotides in a 5'$\rightarrow$3' manner.[9] The DNA template can only be synthesized in a 5'$\rightarrow$3' manner, therefore when it reaches the other template strand that is orientated in a 3'$\rightarrow$5' manner, a second DNA polymerase comes in and begins synthesizing the DNA in small fragments that are later joined together by an enzyme called ligase.[9] This process of DNA replication is an extremely accurate process with an error rate as low as 1 out of every 10^{10} bases.[11] The high fidelity of these enzymes is the result of several contributing factors: the intrinsic ability of the replicative polymerases to select the correct nucleotide, their ability to recognize and excise misincorporated nucleotides, and the activity of post-replicative mismatch repair.[11] However, the features that contribute to their high fidelity, also make them extremely sensitive to damage. If the DNA replication process is occurring, and the polymerase encounters a lesion caused by a carcinogen, in most cases it will not continue to synthesize, therein inhibiting replication, forcing the cell down the pathway of apoptosis.[9, 11]

A carcinogen is any substance, both natural and synthetic, which promotes carcinogenesis, the formation of cancer.[12] Chemical carcinogens are responsible for most cancer deaths in the United States.[2] There are two major classes of chemical carcinogens that are currently recognized. Direct-acting carcinogens are chemicals that bind to DNA and act as mutagens. The second class are procarcinogens, which must be converted metabolically to become active carcinogens which then bind to DNA and act as mutagens.[9] There are several key characteristics that contribute to a substance being carcinogenic. The first characteristic is that the substance is electrophilic and or can be metabolically activated to become an electrophile. Electrophiles are electron-seeking

molecules that commonly form addition products, referred to as adducts, with cellular macromolecules including DNA, RNA and proteins.[12] Some examples of direct-acting electrophilic carcinogens include sulfur mustards and ethylene oxide. Some examples of procarcinogens that need to be metabolically activated to cause damage include polycyclic aromatic hydrocarbons, aromatic amines, N-nitrosamines, aflatoxins, and benzene. These molecules all have the ability to form adducts on nucleic acids and proteins which is a common property of inherent electrophilic and or metabolically activated human carcinogens. [12]

A second characteristic commonly seen in carcinogens is that they are genotoxic, which means that they are able to induce DNA damage, mutation, or both. DNA damage can be spontaneous from the source via errors of nucleic acid metabolism or can be induced by endogenous or exogenous agents.[12] In some cases the exogenous agents may also be generated endogenously, such as formaldehyde and acetaldehyde and UV radiation, producing a background level of DNA damage.[12] Examples of DNA damage include: DNA adducts, which occurs when a molecule is bound covalently to DNA, DNA strand breaks which are breaks in the phosphodiester bonds, DNA crosslinks which occur when a chemical agent reacts with to nucleotide bases and forms a covalent bond between them, and DNA alkylation.[12] The DNA damage by itself is not a mutation and generally does not alter the linear sequence of the nucleotide bases in the DNA.[12] A mutation is a change in the DNA sequence and usually arises as the cell attempts to repair the DNA.[9,12]

A third characteristic is the ability of the substance to alter DNA repair mechanisms or causes genomic instability.[12] Normal cells avoid detrimental mutations by replicating their genomes with high precision.[12] However, this precision of the replication process can vary depending on which polymerase is involved, introducing the possibility of error.[12] The nature of

the error, the flanking sequence, the presence of DNA damage, and the ability to correct errors all affect the outcome of this process.[12] As a consequence, defects in processes that determine DNA-replication fidelity can confer strong mutator phenotypes that result in genomic instability.[12] Thus, carcinogens may act not only by producing DNA damage directly, but also by altering the processes that control normal DNA replication or repair of DNA damage.[12] Examples include the inhibition of DNA repair by cadmium and formaldehyde. [12]

There are three stages involved in chemical carcinogenesis (figure 2).[13] These are defined as initiation, promotion and progression. Each of these stages is characterized by morphological and biochemical modifications and result from genetic and/or epigenetic alterations.[13,14] These genetic modifications include: mutations in genes that control cell proliferation, cell death and DNA repair for example, mutations in proto-oncogenes and tumor suppressing genes.[14] The first stage of carcinogenesis is called initiation which is caused by irreversible genetic changes which predisposes normal cells to transform into malignant cells.[14] The normal cell is not yet neoplastic or in other words does not possess the ability to divide more than they should or lack the ability to die, but has taken its first step towards this.[13-15] The cell has undergone mutations that induce proliferation but not differentiation.[13-15] DNA damage has been well established as the event which kick-starts chemical carcinogenesis.[13] Initiation is a rapid, irreversible phenomenon, and cellular proliferation is essential for this stage.[13-15] If cellular division occurs before DNA repair systems can carry out their process, then the injury becomes permanent and irreversible.[13-15] Initiation is an additive process, neoplastic development depends on the carcinogenic dose, increasing the dose increases the incidence and the multiplicity of resultant neoplasia and reduces the latent period of its manifestation . [13-15]

The second stage is the promotion, which is when cell proliferation in susceptible tissues continues and enhances alterations in genetic expression resulting in changes in cellular growth control. [13-16] Cells are only affected in this stage if they have already been stimulated to divide, are undifferentiated, and have survived apoptosis, so they can contribute to the instability between growth and cell death which leads to neoplasia. [13-16] The third and most prominent stage in carcinogenesis is progression. [13-16] Progression is characterized by irreversibility, genetic instability, faster growth, invasion, metastasis and changes in the biochemical, metabolic and morphological characteristics of cells. Angiogenesis, the development of new blood vessels to supply nutrients to the newly growing cells, is essential to neoplastic progression. [13-16] The acquisition of an angiogenic phenotype precedes the development of characteristics that contribute to malignancy and its inhibition delays neoplastic development. [13-16]
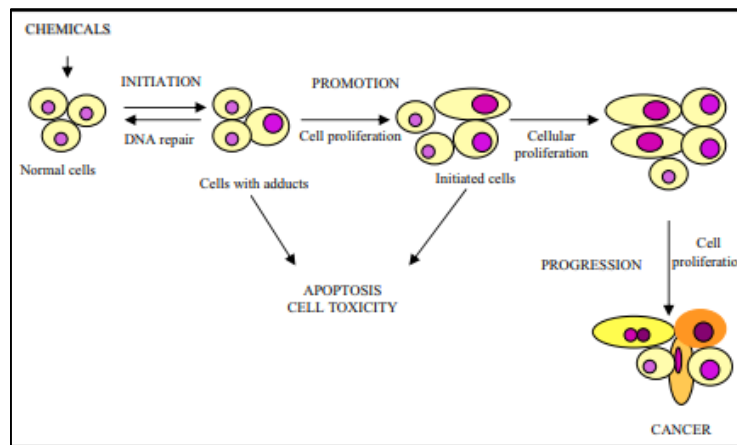

Figure 2. Chemical carcinogenesis stages and the occurrences involved in each one.

Due to the importance of DNA replication, the maintenance and integrity of our DNA is crucial. However, as mentioned before he DNA within our cells is constantly being damaged by chemicals, and the avoidance of carcinogens is almost impossible.[17,18] To combat the cellular damage our cells have developed several repair mechanism to fix and or tolerate the damage to allow cellular process to continue. There are two general categories of repair systems based on

the way that thy function.[9] The first are direct reversal repair systems which correct damaged areas by reversing the damage these types of repairs are carried out by mismatch repair carried out by DNA polymerases and the repair of UV-induced pyrimidine dimers through photoreactivation.[9] The second category involves excision repair systems that excise out the damaged area and then repair the gap by new DNA synthesis. One common type of excision repair is base-excision repair.[9]

Direct reversal mismatch repair is carried out by DNA polymerase proofreading. Base-pair substitution mutations occur every 10^{-7} to 10^{-11} per generation.[9] However, DNA polymerases insert an incorrect nucleotide at a frequency of 10^{-5}.[9] The difference between the mutation rate, and the incorrect nucleotide incorporation rate can be attributed to the 3'-to 5' exonuclease proofreading domain within the polymerase.[9] When an incorrect nucleotide is inserted, the proof-reading domain detects the mismatched basepair and corrects the area by backtracking to remove and replace the wrong nucleotide and then continue on with the synthesis.[9] Base excision repair occurs when the DNA damage only affects one of the two strands. In these cases, the damage can be cut out and the undamaged strand can be used as a template for the insertion of a correct nucleotide.[9] There are two common types of excision repair.[9] The first is base excision repair, which occurs when a repair glycosylase enzyme removes the damaged base from the DNA strand by cleavage of the bond between the base and the deoxyribose sugar.[9] Following that, more enzymes will be recruited in to cleave the sugar-phosphate backbone before and after the now base-less sugar, resulting in a gap in the DNA strand.[9] This gap is filled with the correct nucleotide complementary to the template strand via a DNA repair protein and DNA ligase.[9]

However some lesions are large enough that block the replication machinery from proceeding past the lesion because of the compact nature of the catalytic active site of most DNA polymerases.[18] If the replicative machinery stalls, and cannot proceed on past the lesion it can

become lethal.[18] In order to prevent cellular apoptosis from constantly occurring whenever a bulky lesion is detected cells undergo damage tolerance using a mechanism called translesion synthesis (TLS). [18] TLS utilizes specialized DNA polymerases to bypass the damage, which normal DNA polymerases cannot do, they allow cells to cope with unrepaired DNA damage by promoting replication through lesions.[18] What makes these TLS polymerases unique compared to the normal replicative enzymes is that they possess enlarged active sites that are capable of accommodating the damaged or distorted templates.[17-18] However, the use of the proteins is often a last-resort effort because they are extremely error prone and carry an increased risk of mutagenesis.[17,18] The increased risk of mutagenesis is due in part to the damaged bases are often non- or mis-instructional of the genetic code and because the structural adaptations that allow lesion replication also result in reduced fidelity when copying undamaged DNA.[17,18]

Humans have four Y-family DNA polymerases – Pol η, Pol ι, Pol κ, and Rev1 – each with a unique DNA damage bypass and fidelity profile.[17,18] Polη, for example, is unique in its ability to replicate through Ultraviolet (UV) light induced cross-linked DNA.[17,18] Because of the involvement of Pol η in promoting error-free replication through UV-induced cross-links, its inactivation in humans causes the variant form of xeroderma pigmentosum, a genetic disorder characterized by a greatly enhanced predisposition to sun-induced skin cancers.[17,18] Pol κ, on the other hand, is specialized for replicating DNA damaged with the carcinogen found in cigarette smoke benzo[a]pyrene.[17,18] Presumably, the differences in the ability of Y-family polymerases to replicate across specific lesions are a result of differences in structure. [18,19]

The Y-family polymerases all possess an overall right-handed configuration similar to the normal replicative enzymes (Figure 3).[19] The 'palm' domain contains the key carboxylate residues needed for catalysis, but what differentiates them from other replicative enzymes is that they possess a much larger active site pocket that can accommodate bulky or distorted DNA templates.[19] The fingers and thumb domains used to detect and select the fit of the incoming nucleotides are typically shorter in these Y-family polymerases, and make fewer contacts with both the
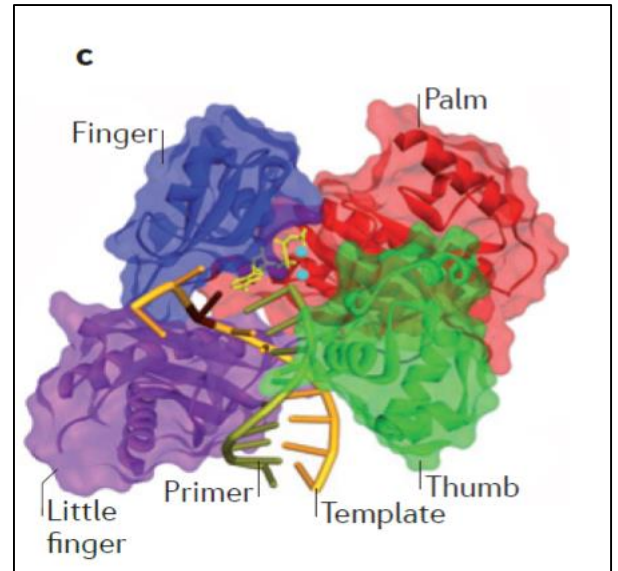
Figure 3. Crystal Structure of Y Family Polymerase Eta. The main domains are color coded (palm = red, thumb = green, finger = blue, exonuclease (Exo) = purple, amino-terminal domain (N) = yellow).[19]

template and incoming nucleotide contributing to the reduced fidelity observed in these molecules.[19] Another unique feature that the Y-family polymerases possess is a little finger domain, most often referred to as the polymerase-associated domain (PAD), that provides stability when replicating the DNA.[19] The most critical feature that Y-family polymerases lack is a 3'→5' proof-reading exonuclease domain which is what results in an extremely error prone replicative process, with error incorporation rates of up to one in ten nucleotides.[19] However, the mutations induced through this mechanism are less harmful than the potentially lethal alternative of an incompletely replicated DNA strand.[19]

My area of research focuses on the TLS polymerase protein, DNA polymerase eta (pol η).[20,21] DNA pol η, is one of the best characterized translesion synthesis polymerases. [20,21] It has been shown that pol η is specifically optimized for bypassing the most profound type of DNA lesion, cyclobutane pyrimidine (CPD) dimers caused by UV radiation of sunlight. [20,21] The damage

caused by ultraviolet radiation catalyzes covalent linkages between adjacent pyrimidines in DNA, which results in dimers which create roadblocks to most DNA polymerases. [20,21] Human pol η is able to perform replication past these bulky thymine dimers because of its larger active site that can accommodate the DNA lesion distortion. [20,21] Pol η is the only one of the fifteen human DNA polymerases in which defects are unequivocally associated with cancer.[11] Research has shown that humans who have a defective *POLH* gene, which encodes pol η, are effected by a variant form of human xeroderma pigmentosum syndrome (XPV). [20,21] XPV is an inherited disorder caused by a deficiency in human pol η and is typically associated with an increased occurrence of sunlight-induced skin cancers. [20,21]

More recently the Y-family polymerases have been gaining interest because of their potential involvement in chemotherapeutic resistance.[21] DNA damaging chemotherapy is typically the first line of treatment for certain cancers, such as testicular and colorectal cancers.[21] One common type of chemotherapy is carried out with the use of genotoxic agents. These types of drugs either bind to DNA or indirectly damage DNA by affecting enzymes involved in replication, and ultimately leading to induced apoptosis.[22] This type of treatment is effective at targeting the disease because proliferating cancer cells undergo replication and division at a much higher rate than healthy cells.[22] There are several types of genotoxic compounds used for treatment; including alkylating agents, enzyme inhibitors and intercalating agents.[22]

Enzyme inhibitors work by blocking replication and inhibiting enzymes involved in the process. One example of enzyme inhibitors used are topoisomerase I inhibitors that interfere with strand separation during DNA replication.[22] Enzyme inhibitors are less frequently used because their mutagenic properties make them carcinogenic and open the door for the potential of developing secondary cancers.[22] Alkylating agents work by altering the nucleotides within DNA

resulting in an interference with DNA replication and transcription leading to mutations within the genetic code.[22] Alkylating agents work by reacting with the proteins that bond together to form the very delicate double helix structure of a DNA molecule, adding an alkyl group to some or all of them.[22] This prevents the proteins from linking up as they should, causing breakage of the DNA strands and, eventually, the death of the cancer cell.[22] Some examples of alkylating agents include nitrogen mustard, alkyl sulfonates, and triazines. Intercalating agents bind within the groves of the DNA helix and interfere with polymerase activity during replication and transcription.[22]

While DNA damaging chemotherapy can be very effective and even curative in the treatment of certain cancers, intrinsic and acquired drug resistance underlies tumor progression and morbidity in many cancer patients.[23] Intrinsic resistance defines a cell state that is inherently tolerant of drug action.[23] This can include the activation of drug efflux pumps or detoxifying processes that effectively reduce intracellular drug concentration.[23] This can also include a change in the recognition or persistence of DNA damage, mediated by an enhanced DNA repair capability, a blunted DNA damage response, or the ability to proliferate in the presence of DNA damage.[23] Mutagenic TLS polymerases underlie two important phenotypes in response to genotoxic chemotherapy.[23] First, they allow for the bypass of modified DNA bases during DNA synthesis, allowing proliferation to continue in the presence of chemotherapy.[23] Second, the low fidelity replication performed by TLS polymerases results in the introduction of inappropriate, non-pairing bases across from modified nucleotides.[23] The bypass function of TLS polymerases is particularly relevant to intrinsic drug resistance.[23] Many tumors, including most pancreatic adenocarcinomas, non-small cell lung cancers, and aggressive brain tumors, as well as most metastatic malignancies, fail to significantly regress following chemotherapy.[23] In these tumors, TLS activity contributes to a drug resistant state by promoting the tolerance of DNA damage.[23]

One chemotherapeutic drug that has shown high-rates of chemotherapeutic resistance is Cisplatin, or cis-diamminedichloroplatinum (II). It is a well-known chemotherapeutic drug that has been used for treatment of numerous human cancers including bladder, head and neck, lung, ovarian, and testicular cancers.[21,24] It is also effective against various types of cancers, including carcinomas, germ cell tumors, lymphomas, and sarcomas.[21,24] The most abundant DNA adduct arising from the reaction between cisplatin and DNA is the intrastrand cross-link between two guanines (Pt−GG) (figure 4).[21,24]
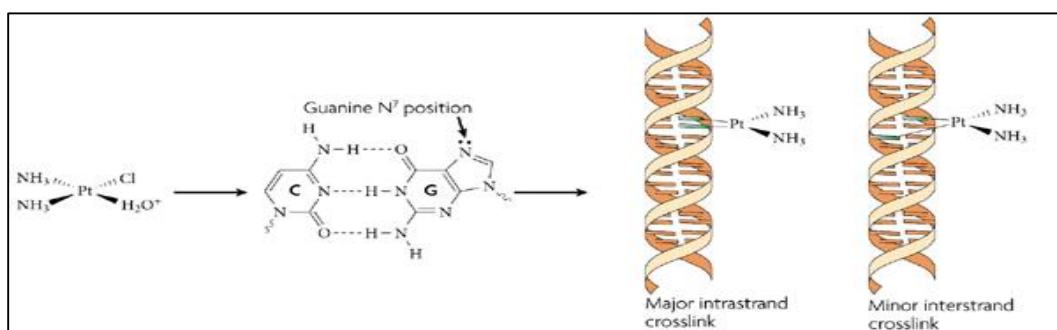

Figure 4. Cisplatin binding to dGTP forming a PT-GG intrastrand and interstrand crosslinks.

These cross-links on the DNA; interfere with DNA repair mechanisms, causing DNA damage, and subsequently inducing apoptosis in cancer cells. [21,24] Though not as cytotoxic as interstrand cross-links, intrastrand cross-links must still be repaired and/ or bypassed.[24] In this regard, human DNA pol η seems to be an important player in the tolerance of unrepaired intrastrand cross-links generated by platinum drugs.[24] However, cancer cells are employing TLS to tolerate the damage caused by the treatment, rendering it ineffective. Research has linked pol η as the main culprit behind cisplatin resistance because the cisplatin–cross-linked guanines (Pt-GG)

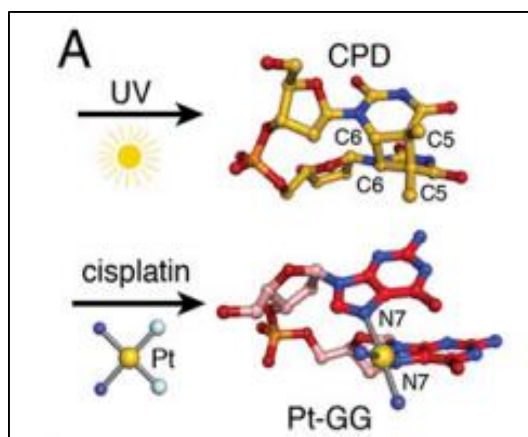are structurally similar to the cyclobutane pyrimidine dimers (CPD) induced by UV radiation (figure 5).[21]

Figure 5. Structures of a thymine dimer (CPD) and a Pt-GG lesion.[15]

Kinetic assays have revealed that kinetic analysis revealed that pol η copies the 3′-dG and 5′-dG sites of a Pt−GG adduct with catalytic efficiencies of 82 % and 35%, respectively relative to unmodified DNA, meaning that it has an affinity for successfully replicating past these bulky cross-links compared to a normal nucleotide within the DNA strand.[24] Another study found that cells derived from xeroderma pigmentosum variant (XPV) patients that do not express functional pol η are considerably more susceptible to cisplatin treatment than cells that express proficient levels of pol η.[24]

Crystal structures have revealed that pol-η can accommodate a bulky Pt-GG DNA crosslink within its active site cleft without any major rearrangement of the enzyme.[21] The specificity of human pol η for a Pt-GG, compared to other DNA polymerases, derives from an active site cleft that is sufficiently open to allow near perfect Watson-Crick geometry of the Pt-GG-dCTP (cytosine) base pair, but is also to accommodate the inclined 5′ guanine of Pt-GG without any steric hindrance.[24] The pol η active site cleft is well poised for catalysis and the incorporation of cytosine opposite the 3′ guanine of Pt-GG (Figure 6).[21] Thus, even though Pt-GG is a larger and

more distorted intrastrand crosslink than a cis-syn thymine–thymine dimer, it does not lead to any substantial agitation of the human pol η active site or affect the ability of the polymerase to carry out replication.[21] The primer terminus is well aligned for the incorporation of cytosine opposite the 3′ guanine of the Pt-GG adduct allowing replication to continue past the lesion.[21] Inhibition of pol η may potentially reduce chemoresistance to platinum-based drugs caused by translesion DNA synthesis.[21,24]
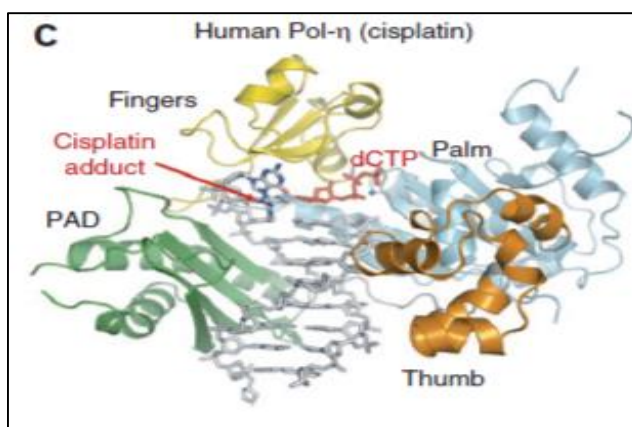

Figure 6. The structure of human Pol-η in ternary complex with the cisplatin intrastrand cross-link (PtGpG) DNA and the incoming dCTP. The PtGpG is shown in gray and blue, and the incoming dCTP is shown in red. The putative Mg2+ ion is shown in dark blue.[21]

Potential inhibitor screening assay against Y-family polymerases

A strand-displacement assay is currently being used as a technique to analyze pol η activity with various potential inhibitors. The way the strand displacement assay works is based on fluorescent reporter strand displacement from a tripartite (three part) substrate containing a quencher-labeled template strand, an unlabeled primer and a fluorophore-labeled reporter.[25] The idea behind it is that if the polymerase is functioning, it will begin to incorporate nucleotides on to the free 3' end of the primer, and as this is occurring it will displace the fluorescent reporter away from the quencher giving off a fluorescent signal.[25] The degree of fluorescent signal is directly proportional to enzyme concentration, and activity. As you increase the protein's

concentration, the level of activity, in this case DNA replication will increase resulting in an increase of fluorescence. So in the presence of an inhibitor, the level of fluorescence would decrease as a result of enzyme inhibition which is what makes this assay useful in screening for compounds. Currently, this method is being employed on another y-family polymerase, DNA polymerase kappa, and several compounds have been found to have inhibitory effects on its activity.[25] The identification of several compounds against polymerase kappa is what provided us a starting point to begin looking for potential inhibitors of pol η.

A study conducted by *Woodgate et al.* identified three compounds that had significant inhibitory effects on polymerase Kappa. The three that they identified were Ellagic acid, Pamoic Acid, and Aurintricarboxylic acid (figure 6).[25] Ellagic acid (EA) is a naturally occurring polyphenolic compound which is found in many fruits, nut galls and plant extracts in the forms of hydrolysable tannins called ellagitannins such as raspberries, strawberries, grapes, pomegranate, black currants, mango, guava, walnuts, almonds, longan seeds and green tea.[26] It is a highly thermostable molecule that
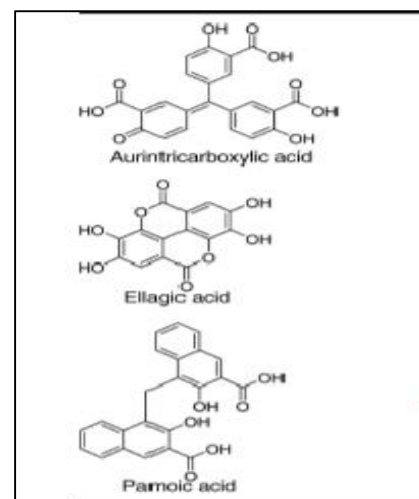

Figure 7. The structure of the compounds used in the current study.

is slightly soluble in water, alcohol, and ether. Structurally, presents four rings representing the lipophilic domain, four phenolic groups and two lactones, which form hydrogen-bonds sides and act as electron acceptors respectively, and that represent the hydrophilic domain.[26] Evidence suggests EA's mode of action is that binds covalently to DNA and induces DNA single-strand breaks and inhibits the replicative and repair synthesis of DNA.[26]

Pamoic acid (4-[(3-carboxy-2-hydroxynaphthalen-1-yl methyl]-3-hydroxynaphthalene-2-carboxylic acid) has been used in formulations of several drugs as pamoate salts.[27] It is

commonly used pharmaceutical salt pamoic acid to activate GPR35, an orphan G protein-coupled receptor (GPCR but has been identified as a potential inhibitor of polymerase Kappa through unknown mechanisms.[27] The third compound, Aurintricarboxylic acid showed the strongest inhibitor effect against pol kappa. Aurintricarboxylic acid can be prepared by the condensation of formaldehyde with salicylic acid in the presence of nitrite-containing sulfuric acid.[28] Its mode of inhibition is believed to be due to the fact that it readily polymerizes in aqueous solution, forming a stable free radical that inhibits protein-nucleic acid interactions such as the interaction between DNA polymerases and the DNA base.[28]

The goal of this project was to incorporate the high-throughput screening assay described above to identify additional l drug inhibitors against pol η. The first step was to transform the gene that encodes for pol η into a competent, *Escherichia coli* (*E.coli)* cell line and grow the bacterium up using the technique of recombinant protein expression. Once protein of interest, pol η, was expressed, it needed to be purified. Protein purification is vital for the characterization of the function, structure, and interactions of proteins. The various steps in the purification process may include cell lysis, separating the soluble protein components from cell debris, and finally separating the protein of interest from product- and process-related impurities using various types of chromatography. The results from this research lead to the optimization of the strand-displacement assay to screen for inhibitors for pol η. Ellagic acid was found to have a potential inhibitory affect against pol η.

Methodology

Protein Expression of Polymerase Eta (Pol η)

Recombinant protein expression is performed by placing the gene that encodes the pol η protein into a host organism that will be used to grow the protein. Wild-type pol η was obtained

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using standard *E. coli* protein expression. The plasmid DNA that encodes for pol η was obtained from Dr. Penny Beuring's lab at Northeastern University. The plasmid was designed to work on the lactose operon repression system. The lactose operon is found naturally in *E. coli*, and when genetically engineered into a plasmid it contains a specific sequence ahead of the gene of interest, in this case pol η, to which a protein repressor can bind. The repressor averts transcription factors and RNA polymerase from binding to the plasmid DNA and inhibiting the production of the protein.[29] Utilizing a lactose-operon system, *E. coli* can be induced using isopropyl-β-D thiogalactopyranoside (IPTG) to control its expression of the gene of interest.[29] The IPTG binds to the repressor, causing a conformational change that removes the repressor from the DNA.[29] Once the repressor is away from the DNA, pol η can be transcribed and translated into protein. This technique is used to ensure a high yield of protein expression.

The plasmid was also manipulated so that pol η was expressed fused to multiple histidines and a glutathione S-transferase (GST) tag at the end terminal of the protein. The GST-histidine tag (his-tag) serves purpose for use in affinity chromatography, a method of purification used to extract proteins from cell lysates. In addition to taking advantage of the lactose operon and his-tag, the plasmid containing pol η contained a gene for kanamycin resistance. Kanamycin is a broad-spectrum antibiotic that causes breakdown of the cell wall of bacteria. Bacteria that do not have resistance to the antibiotic will not grow in culture. Therefore, the kanamycin resistance gene is expressed actively to prevent the growth of unwanted organisms and to allow for the selection of *E. coli* cells that have successfully taken up the pol η plasmid.

The Pol η plasmid was transformed in BL21-Gold *E. coli*. The cells were streaked for single colonies onto an LB Agar plate, and grown overnight at 37°C. A single colony containing genetically pure E. coli was grown in 10 mL sterile TY Medium broth overnight with shaking. The

resulting growth was divided into four 1 L flasks of sterile TY Medium broth, and grown at 37°C with shaking to an absorbance of 0.6 at 595 nm. The absorbance reading indicated sufficient growth for Pol η production. The flasks were cooled to 18°C and induced with IPTG, and left to shake overnight to produce Pol-η. The next day, the 4 L of broth and *E. coli* were centrifuged at 4°C for 35 minutes at 5000 RPM, respectively, to pellet the *E. coli* cells containing the protein of interest, Pol-η.

Extraction of Pol-η from *Escherichia coli* Cells

The cell pellet was resuspended in a buffer solution (Buffer A) containing 50 mM Tris (pH 8.0), 300 mM NaCl, 20mM imidazole, 10% glycerol, 10mM 2-mercaptoethanol. Protease inhibitor cocktail tablet was added to prevent degradation of Pol η. The resuspended cells were sonicated with high frequency sound waves to lyse open the cell wall and extract the protein. Sonication cycle is as follows: 10 second burst, 30 second break, 10 second burst. This process was repeated 4 times, with a 2 minute break in between each cycle, to ensure proper cell lysis. Following sonication the cell solution was spun-down using an ultracentrifuge. The cells were spun at 18,000RPM for 40 minutes to pellet the cellular debris, and collect Pol η in the lysate.

Purification of Pol η

The purification of Pol η was achieved through a two-step process using a GE Healthcare sciences 5mL HisTrap Column with fast protein liquid chromatography (FPLC), following a Biorad gravity glutathione Sepharose column to obtain pure, untagged wild-type Pol η.

Affinity Chromatography

Nickel columns are used for immobilized metal affinity chromatography (IMAC) for the purification of recombinant proteins that contain a histidine tag. The histidine residues in the tag

bind to the vacant positions in the coordination sphere of the immobilized nickel ions in the column.[30] When the filtered lysate is loaded on the column, the His-tagged protein is bound and other unwanted proteins pass through the column matrix.[30] In the case of column chromatography two buffers are used, typically denoted as buffer A and buffer B. Buffer A is used as a wash buffer to ensure proper purification of the protein is achieved, and no unwanted proteins remain, then Buffer B is used as an elution wash to strip the protein of interest from the column. In the case of nickel chromatography, Buffer B contains a high imidazole concentration to strip the protein from the column. Imidazole is a chemical that structurally similar to that of the histidine molecule, so at high concentrations, it will compete for the empty positions in the nickel ions, essentially forcing the histidine tags out, releasing it from the column.[30] All washes during the column are collected in fractions for analysis.

Fast Protein Liquid Chromatography (FPLC)

Fast protein liquid chromatography (FPLC) is a form of medium-pressure chromatography that uses a pump to control the speed at which the mobile phase passes through the stationary phase.[31] In terms of this experiments, the stationary phase was the nickel column, and the mobile phases were Pol n, followed by buffer A and buffer B. FPLC systems generally consist of a pump, a UV detector, a conductivity meter, and a fraction collector and operate at pressures of ~3,500 psi (24 MPa).[31] As the system is running, a chromatograph is generated to visualize the purification in real-time. It graphs the UV absorption vs the concentration of elution buffer. Proteins are large biomolecules that contain several aromatic rings that have the ability to absorb light, an absorption signal is detected around 280nm when it is eluted from the column.[31]

Purification Procedure

The purification of Pol n was run at 4°C. The first step was to prepare the two buffers; Buffer A and Buffer B. Buffer A contained 50 mM Tris (pH 8.0), 300 mM NaCl, 20mM imidazole, 10% glycerol, 10 mM 2-mercaptoethanol. Buffer B was prepared identically, except instead it had a 200 mM imidazole concentration to ensure successful elution from the column. Following buffer preparation the sample, the filtered Pol η lysate underwent a 10-fold dilution, and 200 mL of the lysate was loaded onto the column. Once the protein was loaded, on to the column, the solution probes were placed into buffers A and B and a linear gradient wash was carried out by the FPLC. Fractions were collected and analyzed via SDS gel electrophoresis to identify which fractions contained Pol η.

SDS-PAGE is a polyacrylamide gel electrophoresis (PAGE) technique that uses sodium dodecyl sulfate (SDS) to coat proteins with a negative charge. The negative charge allows the proteins to migrate in an electric current towards a cathode. The polyacrylamide gel slows proteins in its polymerized matrix, allowing smaller proteins to flow through faster than larger proteins, thus separating the proteins by molecular weight. A bromophenol blue dye (0.125 M Tris-HCl [pH 6.8], 5 mM EDTA, 15% glycerol, 2% SDS, 1% 2-mercaptoethanol, 0.1% bromophenol blue) is added to the samples in the gel in order to track the progress through the gel. Once the dye front has reached the bottom of the gel, the gel is removed and the proteins within are stained with Coomassie Blue. Pol η can be analyzed in this way by comparing the bands loaded in each lane with a molecular weight marker. The cell lysate, and the fractions collected from the FPLC were loaded onto a gel to locate which fractions contained Pol η. All SDS-PAGE was performed using a Bio-Rad Mini-PROTEAN 3 apparatus with 10% polyacrylamide gels in Tris buffer (diluted from 5X stock - 0.125 M Tris base, 0.96 M glycine, 5.0 g SDS).

The next step in the purification process was to remove the GST/His-tag from the protein to prevent any unwanted interactions occurring in future assays. This was done via protein digestion to cleave the His-tag, and glutathione sepharose chromatography to recover the pure-untagged protein. The fractions containing purified Pol η were pooled together and loaded onto a GST column, and an enzyme called PreScission Protease was used to digest and cleave the GST-His-tag from the end terminal of the protein. PreScission Protease is a genetically engineered enzyme made up of human rhinovirus 3C protease and GST (Viljanen, Larsson, and Broo; 2008). The enzyme's protease domain site-specifically cuts between the Gln and Gly residues of LeuGluValLeuPheGln/GlyPro, a peptide sequence cloned in between GST and pol η. Pol η and precision protease was left to incubate in the column overnight to ensure cleavage and proper binding of the GST tag to the matrix of the column. The following day, the flow through, which should only contain untagged Pol η, was collected by FPLC and visualized via SDS- gel electrophoresis.

Quantification of Pol η

To determine the concentration of protein that was purified a Bradford assay was performed. The principle of this method is based on the proportional binding of the dye Coomassie Brilliant Blue-G250 to proteins.[32] Within the linear range of the assay, the more protein present, the more Coomassie binds.[32] Furthermore, the assay is colorimetric; as the protein concentration increases, the color of the test sample becomes darker.[32] Coomassie Blue dye absorbs at 595 nm. The protein concentration of a test sample is determined by comparison to that of a series of protein standards known to reproducibly exhibit a linear absorbance profile in this assay.[32] The protein standard that was used Bovine Serum Albumin (BSA).

Five standard solutions of BSA were prepared with deionized water in clear plastic cuvettes with the following concentrations; 2 µg/mL, 4 µg/mL, 6 µg/mL, 8 µg/mL, and 10 µg/mL. Biorad Coomassie Brilliant Blue-G250 dye reagent was added, and the mixture was inverted and then incubated for five minutes. After the incubation period the absorbance was taken at 595nm using the spectrophotometer. This experiment was run in triplicate, and the absorbance values of each trial were recorded.

The preparation of samples to determine the concentration of Pol η was down by a guess and check method. Several solutions with "X µL" of Pol η were pipetted and put into "**x** µL" amount of dH_2O so that the total volume was 800uL, and then 200µL of dye was added to bring the volume to 1mL. After adding the dye, the samples were left to incubate at room temperature for 5 minutes, and then the absorbance was taken at 595nm. If the absorbance value did not fit within the range, then a new sample of protein was made according to the rule that if the absorbance reading was too high, then a more dilute sample was made, if it read too low, then a more concentrated solution was made until it fit the range. Once an absorbance value was detected that fit within the standard range, it was plotted on the graph to determine the concentration.

Polymerase Fidelity Assay

To determine if the Pol η that was purified is active, a polymerase fidelity assay, called the primer-template extension was executed. Template-primer insertion is accomplished in vitro by creating a synthetic DNA template substrate, with a 4 nucleotide overhang on the template stand and a free 3'OH group on the end of the primer that will allow for nucleotide incorporation complementary to the template when nucleotides and polymerase η are added (Figure 8). The products of the reaction will be separated and visualized using denaturing polyacrylamide gel electrophoresis, and visualized using a laser imager.
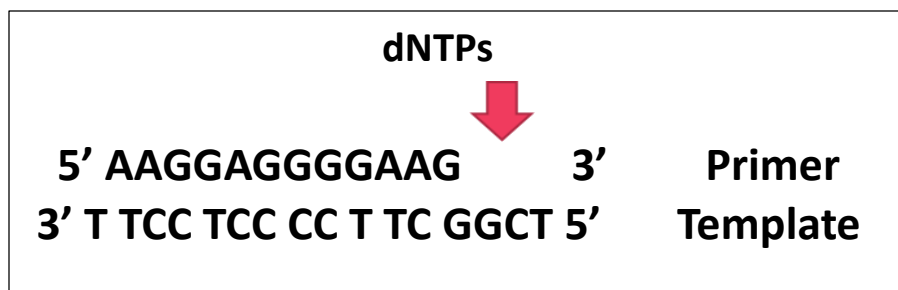
Figure 8. Diagram of the polymerase fidelity assay nucleotide incorporation.

The DNA substrate used was a 13-mer primer/ 18-mer template pair from Integrated DNA Technologies with a 5'-CY5 fluorophore for visualization in a polyacrylamide gel. The reaction conditions were as follows: 20 nM Pol η, 200nM oligonucleotides, 100 μM dNTP (deoxy-nucleotide), 25 mM MgCl₂, 250 mM Tris-HCl (pH 8.0), 200 mM NaCl, and 10 mM dithiothreitol (DTT). The final volume of each mixture was 10 μL. The reactions took place at room temperature for 30 minutes, and then were stopped with bromophenol blue in deionizing formamide. The reaction products were separated using a 40% polyacrylamide gel. The reaction products of Pol η synthesis across from the 18-mer template were visualized using a Bio-Rad Pharos FX plus Molecular Imager and Bio-Rad Quantity One software. The activity of pol η was tested on a time scale, ranging from 30 seconds up until 30 minutes. Human pol η was incubated with the DNA substrate and nucleotides (dNTP's), and then the reaction was quenched at the desired time intervals to view the activity of the polymerase.

Strand-Displacement Assay

The strand-displacement assay was also used in an attempt to identify potential drug inhibitors of DNA pol η. The way the strand displacement assay works is based on fluorescent reporter strand displacement from a tripartite (three part) substrate containing a quencher-labeled template strand, an unlabeled primer and a fluorophore-labeled reporter.[14] The idea behind it is that if the polymerase is functioning, it will begin to incorporate nucleotides on to the free 3' end

of the primer, and as this is occurring it will displace the fluorescent reporter away from the quencher giving off a fluorescent signal (figure 9).
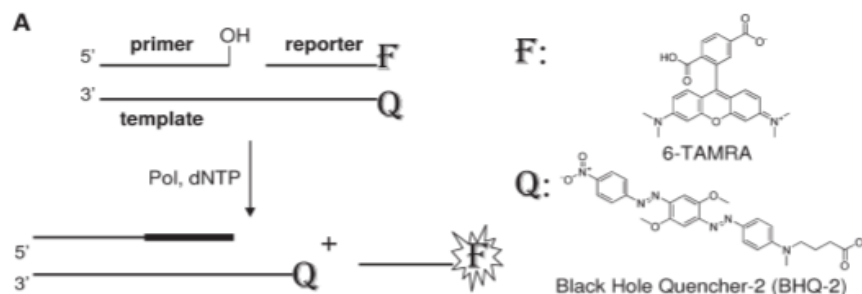

Figure 9. Strand displacement DNA synthesis assay. F and Q denote 6-TAMRA and Black Hole Quencher-2 (BHQ-2), respectively. Chemical structures of 6-TAMRA and BHQ-2 used in the present study are also provided.[14]

The strand displacement assay was run in a 96-well plate using a time scale ranging from 0 seconds up until 50 minutes to view the activity of pol η. The reactions were prepared in 96 well plates. The reaction conditions were as follows: '*X*' nM Pol η, 770mM DNA substrate mix, 400 μM dNTP (deoxy-nucleotide), 25 mM MgCl$_2$, 250 mM Tris-HCl (pH 8.0), 200 mM NaCl, and 10 mM dithiothreitol (DTT). *X*= (50 nM, 100 nM, 200 nM Pol η). All reactions were quenched with EDTA, and fluorescence data was recorded using a microplate reader set to a 525nm excitation and 595 emission.

Strand displacement assays run to test for inhibitor were run in 96 well plates under the following conditions;100 nM Pol η, 770mM DNA substrate mix, 400 μM dNTP (deoxy-nucleotide), 25 mM MgCl$_2$, 250 mM Tris-HCl (pH 8.0), 200 mM NaCl, and 10 mM dithiothreitol (DTT) and 'X' amount of inhibitor compound. X= (20μM- 200μM concentration range). Compounds tested were Ellagic acid, Pamoic Acid, and Aurintricarboxylic acid all from Sigma Aldrich. The reactions were incubated at room temperature and quenched with EDTA after 35

minutes. Plates were then analyzed, and fluorescence data was recorded using the microplate reader set to 525nm excitation and 595nm emission.

Results

Protein Expression, Purification and Quantification.

Several rounds of protein expression and purification were performed as needed to obtain large, homogenous amounts of pol η. Figure 10 shows the SDS-PAGE gel of the fractions collected from the FPLC nickel Histrap purifications. Purified pol η was present in lanes 8-12 around the 77 kDa mark as expected. The chromatograph generated from the UV detector during FPLC purification is shown in Figure 11. The peaks present at 280nm indicate homogenous protein coming off of the column.
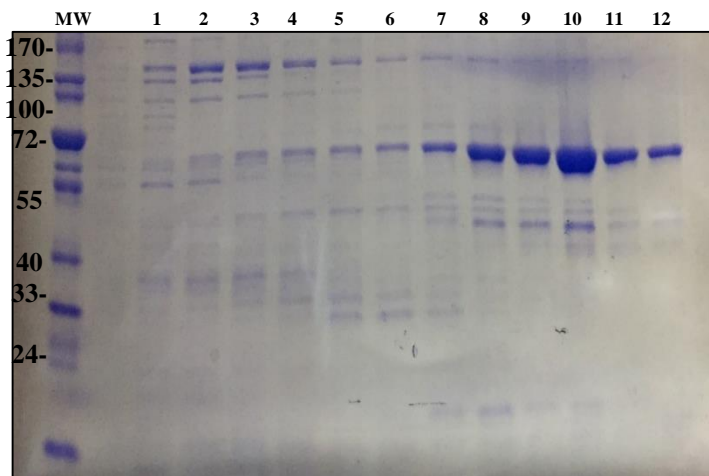

Figure 10. SDS-PAGE gel for determining the purity of Pol η. Lane MW- molecular weight marker (labeled in kDa). Lane 1-12 fractions collected from FPLC purifications. Pol η was found in lanes 8-12 at ~77kdA.
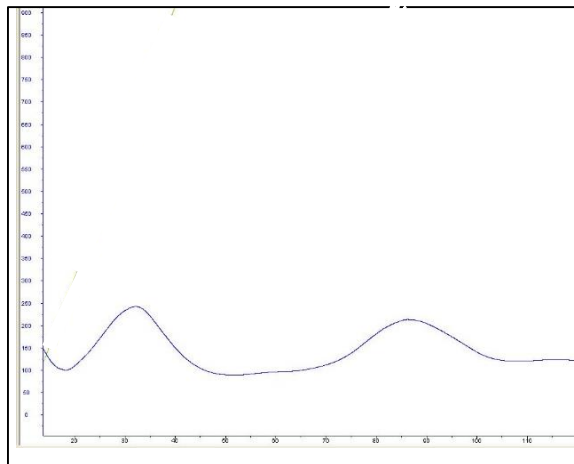

Figure 11. Chromatograph generated during FPLC purification. The presence of protein is indicated by the two peaks at 280nm. The X-axis depicts the volume of buffer eluted, and the Y-axis depicts the relative absorbance in mAU.

Figure 12 shows the SDS-PAGE of the protein digest to get visual confirmation that precision protease cleaved the tag off of pol η. The presence of two bands indicates one belonging to pol η and the other to the GST/His-tag. Figure 12 shows the SDS-PAGE of pure untagged polη.
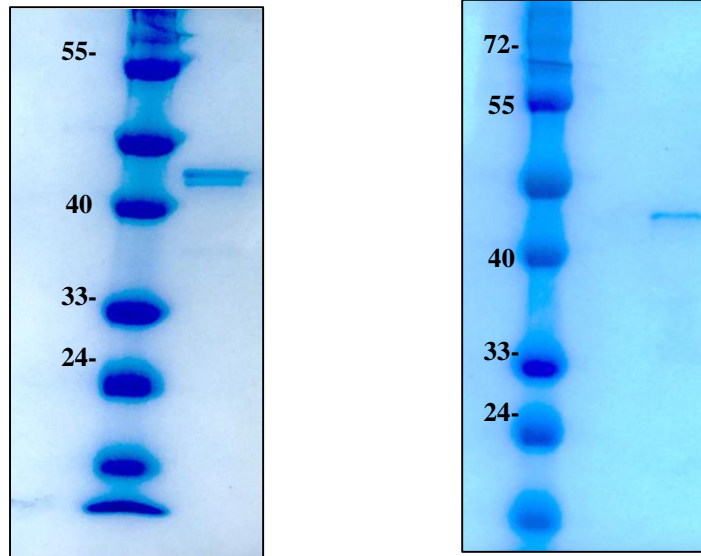

Figure 12. SDS-PAGE gel of protein digest (left) and Pol η after GST Column (right). Lane 1 (left) shows two bands present indicating successful cleavage of the tags. Right- Lane 1 displays the flow through containing untagged Pol η after protein digestion, at ~48kDa.

A plot of absorbance verses concentration was made and the linear standard curve was determined to have an equation of y= 0.0638x +.0394, with an R^2 value of .9996. The standard curve generated from the Bradford assay to determine the concentration can be found in figure 13.
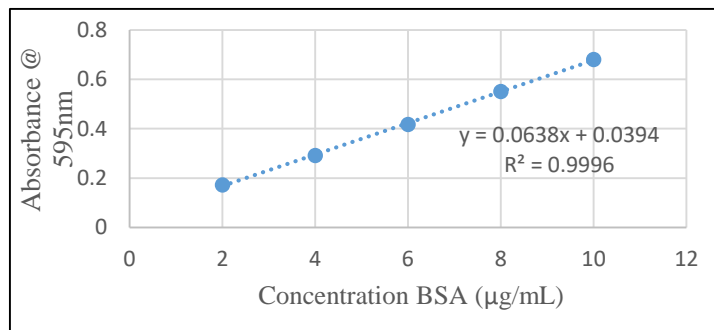

Figure 13. Standard curve generated from the average absorbance of the BSA standards. Equation y = 0.0638x + 0.0394 R² = 0.9996.

Using the equation generated from the standard curve above, the concentration of purified pol η was determined to be 900ug/mL or .188mM of pol η.

Single-Base Nucleotide Primer Assay

One polymerase assay was successfully performed to determine nucleotide incorporation by pol η. The assay was run over the course of 30 minutes, with quenching of the reaction at 1 minute, 2 minutes, 5 minutes, 10 minutes, 15 minutes, 25 minutes and 30 minutes. The results can be seen in Figure 14. Despite the poor gel resolution, shifted bands can be seen in lanes 5, and 6 indicating nucleotide incorporation by DNA pol η. The bands underneath the DNA starting template band indicate impurities within the DNA substrate. It can be visualized that full extenstion onto the primer was obtained at 25 minutes.
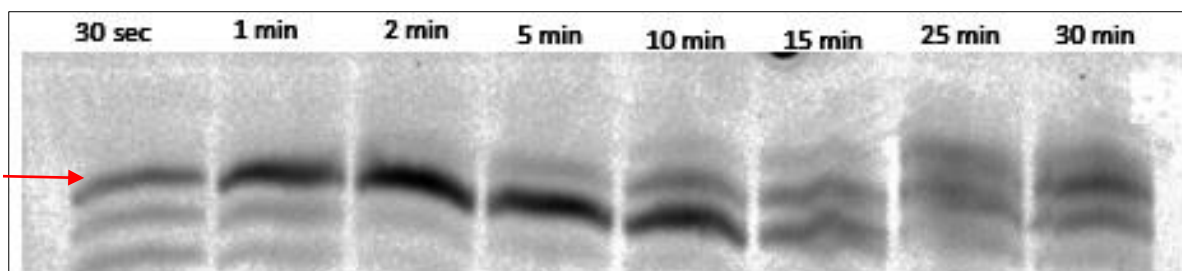

Figure 14. Primer template extension assay over the course of 30 minutes. The red arrow indicates the start of the primer. The red arrow indicates the primer band of the primer. Nucleotide incorporation above the primer can be seen starting at 5 minutes, and full extension is reached at 25minutes. The banding shown underneath is due into impurities in the DNA substrate.

Strand Displacement Assay

To determine the optimal protein concentration for our strand displacement assay, varying concentrations of pol η were tested while keeping other parameters constant. The strand displacement assay was run in a 96-well plate using a time scale ranging from 0 seconds up until 50 minutes to view the activity of pol η. This experiment was carried out using three different concentrations of protein: 50 nM, 100 nM, and 200 nM pol η. The results can be seen in Figure 15. The black line symbolizes the 50 nM pol η, the blue lines symbolizes the 100 nM pol η, and

the green line symbolizes the 200nM pol η. Full extension was obtained at around 40 minutes, indicated by the presence of a plateaued line. Figure 16 displays the data gathered from the strand displacemnet assay when ellagic acid was introduced into the DNA-polymerase mix. It was determined that increasing the amount of ellagic acid had an inhibtory effect on pol η enzymatic activity indicated by a decrease in fluorescence.The graph plots the relative fluorescence against the log of the inhibitor concentration. Pamoic acid and aurintricarboxylic acid were tested against pol η in the strand displacement assay but did not demonstrate conclusive results.
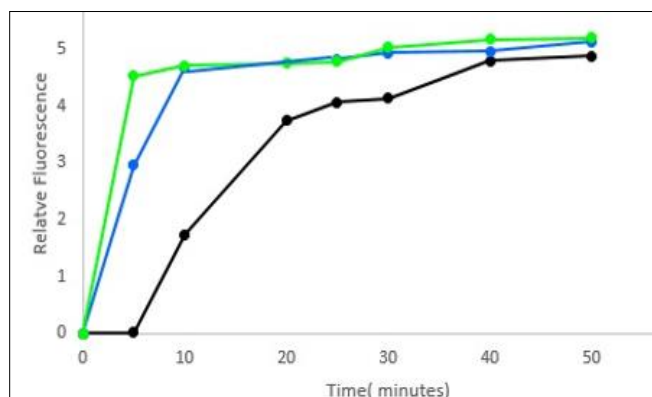

Figure 15. Data collected on the microplate reader presenting various concentrations: 50nM polymerase eta (black), 100nM pol eta (blue), and 200nM pol eta.
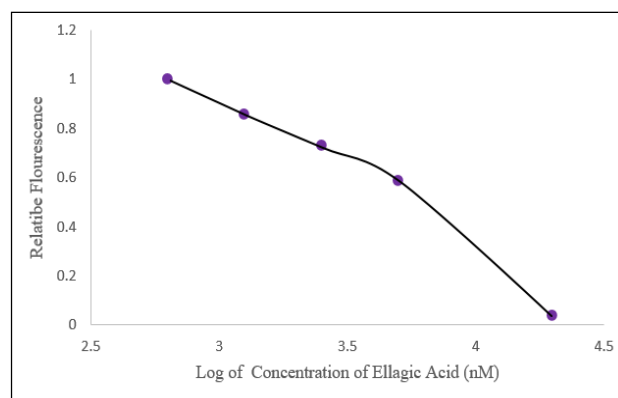

Figure 16. Data collected from the microplate reader displaying the effects of an increasing concentration of Ellagic acid on 100nM of pol η after a 35 minute incubation period, run at 37 °C.

Discussion

The purpose of this work was to implement a real-time reporter-strand displacement assay for DNA pol η to identify potential inhibitors. Inhibition of DNA repair and replication is an established approach to treating cancer, as the elevated expression of DNA repair and damage tolerance proteins can negatively affect patient response to genotoxic drugs.[21] As an example, the Y-family member pol η is overexpressed in the stem cell line of ovarian cancer patients, and this overexpression decreases the effectiveness of cisplatin, shortening the time to tumor recurrence.

The ability to slow the development of resistance and increase efficacy could benefit many patients undergoing chemotherapy with platinum-based drugs.[24]

In my research I developed a working protocol for the expression and purification of pol η. The expression and purification was accomplished by producing wild-type protein using recombinant protein expression techniques, and then programming a purification method on the FPLC using Unicorn[TM] software. The purity of pol η was determined using SDS-PAGE and the quantity of pol η was determined using a spectroscopic technique known as a Bradford Assay. I then characterized the enzymatic activity of pol η by measuring its ability to synthesize DNA. To do this a template-primer extension assay was used to measure the insertion and extension of pol η. This was accomplished *in vitro* by incubating pol η with a fluorescently labelled DNA substrate, and deoxynucleotides. The template-primer extension assay revealed that pol η was able to incorporate nucleotides on to the primer, confirming the enzymatic activity of pol η.

High-throughput screening is a scientific methodology that allows for simultaneous testing for potential drug inhibitors against enzymes of interest. Recently, *Llyod et al.* has developed a high-throughput screen towards Y-family DNA polymerases.[25] The assay is based on fluorescent reporter strand displacement from a tripartite substrate containing a quencher-labeled template strand, an unlabeled primer and a fluorophore-labeled reporter. With this method one can follow the enzymatic activity of these Y family polymerases by quantify the fluorescence intensity using a microplate reader. As a first step of incorporating this screening method to identify potential inhibitors for pol η I performed initial screenings of protein and nucleotides to determine the optimal parameters for fluorescent responses. This was accomplished *in vitro* by incubating varying concentrations of pol η with the fluorescent tripartite DNA substrate and deoxynucleotides over a course of 45 minutes. Through these initial screenings I determined that 100 nM pol η was

a suitable protein concentration for the assay and concluded that full extension onto the primer was achieved at 35 minutes.

With the parameters optimized, I then took to screening the three compounds: ellagic acid, pamoic acid, and aurintricarboxylic acid. The data collected from these initial screenings is preliminary, and a further, more exhaustive study needs to be completed, in which the IC_{50} values would be calculated. However, from the initial screenings, the only compound out of the three that had a noticeable inhibitory effect on pol η enzymatic activity was ellagic acid. This result was expected because a study conducted by *Woodgate at el.* determined that ellagic acid had a significant inhibitory effect on pol η's replicative ability with an IC_{50} value of 0.062 uM. The data obtained from screening pamoic acid and aurintricarboxylic acid against pol η, yielded inconclusive results due to unknown experimental errors. From the data collected, it appeared to have no significant effect on pol η ability however due to a large variation in the numbers gathered from the plate reader no final conclusions can be made. More thorough studies would have to be repeated to make any definite conclusions about the effects of these compounds on Pol eta. *Woodgate at el* screened these compounds against pol η and discovered that they both had inhibitory effects on the polymerases activity. They identified that pamoic acid did not have as large of an effect on pol η activity as ellagic acid did, inhibiting the activity at around 70 uM. Whereas the aurintricarboxylic acid had a simialr potency to that of ellagic acid, with an IC_{50} value of 0.075uM.[25]

Zafar at el. is currently using the strand displacement assay to research the effect of derivatives from N-aryl-substituted indole barbituric acid (IBA), indole thiobarbituric acid (ITBA), and indole quinuclidine scaffolds as potential inhibitors for pol η. Through their studies they have found that 5-((5-chloro-1-(naphthalen-2-ylmethyl)-1Hindol-3-yl)methylene)-2-

thioxodihydropyrimidine-4,6- (1H,5H)-dione (PNR-7-02), an ITBA derivative that inhibited pol η activity with an IC50 value of 8 μM and exhibited 5−10- fold increased specificity for pol η over replicative polymerases.[24] Through molecular docking simulations they hypothesize that PNR-7-02 binds to a site on the little finger domain and interferes with the proper orientation of template DNA to inhibit pol η.[24]

Although the overall goal of identifying compounds that had significant inhibitory effects on pol η enzymatic activity was not accomplished due to experimental set-backs and time constraints, I was able to successfully optimize the reaction conditions and propose a working protocol to monitor the effects of compounds on pol η enzymatic activity to hopefully someday identify inhibitors. Future work on this product includes screening a large library of compounds using this strand displacement assay to identify hits against pol η. Successful inhibition of pol η could render platinum-based treatments more effective by eliminating the tumor cell population largely responsible for tumor relapse and by suppressing the emergence of chemo-resistant tumor cells.[24]

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