Translesion Synthesis of N^2 and C8-Deoxyguanosine Adducts of the Dietary Mutagen 2-Amino-3-methylimidazo-[4,5-*f*]-quinoline (IQ): Participation in Observed Mutagenic Spectra

By

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LIST OF ABBREVIATIONS

Deoxyribonucleic acid ..... DNA

Polycyclic aromatic hydrocarbon ..... PAH

2-naphthylamine (**1.01**) 2-NAPH

simple aromatic amines ..... SAA

4-aminobiphenyl (**1.02**) 4-ABP

o-toluidine (**1.03**) ..... o-tol

2-aminofluorene (**1.04**) 2-AF

polymerases ..... pol

nucleotide excision repair ..... NER

base excision repair ..... BER

mismatch repair ..... MMR

translesion synthesis ..... TLS

heterocyclic aromatic amines ..... HAA

International Agency for Research on Cancer ..... IARC

2-Amino-3-methylimidazo-[4,5-*f*]quinolone (**1.06**) ..... IQ

N-acetyltransferase ..... NAT

2′-deoxyguanosine ..... dG

Eschericia coli ..... E. coli

N-acetyl-2-aminofluorene ..... AAF

small interfering ribonucleic acid ..... siRNA

deoxythymidine-deoxythymidine ..... dTyd-dTyd

xeroderma pigmentosum variant.....XPV

8-oxoguanine.....8-oxo-dG

7,8-diol 9,10-epoxide..... BPDE

γ -hydroxy-1,N^2-propano-deoxyguanosine.....γ-HOPdG

small interfering ribonucleic acid ..... siRNA

mutational frequency .....MF

deoxyribonucleoside triphosphates..... dNTPs

human pol..... hpol

yeast pol ζ.....ypol ζ

C8-dG adduct of 3-nitrobenzanthrone dG-C8-ABA

room temperature.....r. t.

N^2-methyl-dG.....N^2-Me-dG

N^2-methyl(6-benzo[*a*]pyrenyl)-dG ..... N^2-BPdG

N^2-ethyl-dG..... N^2-Et-dG

N^2-methyl(2-naphthyl)-dG..... N^2-Naph-dG

N^2-methyl(9-anthracenyl)-dG .....N^2-Anth-dG

liquid chromatography/mass spectrometry.....LC/MS

CHAPTER I

INTRODUCTION

Background

Cancer has afflicted humans for several millennia. The earliest documented case of disseminated cancer occurred in a Scythian king on the steppes of southern Siberia 2,700 years ago. The oldest written records considered to describe cancer are Egyptian papyri, especially the George Ebers and Edwin Smith papyri written 1600-1500 BC, which depict the pharmacological, surgical, and magical treatments of the time.[1] Diagnosis, treatment, and knowledge of cancer proceeded slowly through the millennia. In ancient Greece "the father of medicine," Hippocrates (460-360 BC) refers to differences between benign and malignant tumors. However, Gabrielle Fallopius (1523-1562) is considered the first to accurately detail the characteristics defining benign and malignant characteristics of tumors. In 1740, the first cancer hospital admitted 8 patients in Reims, France. The citizens' fear of cancer as a contagious disease compelled the hospital to relocate outside the city in 1779. A renowned surgeon, Henri François Le Dran (1685-1770), first speculated cancer spread to distal locations through the lymph nodes. In 1829, Joseph Recamier coined the term "metastasis" to describe the dissemination of cancer. Armand Louis Marie Velpeau (1795-1867) is accredited with postulating the genetic origin of cancer through his hypothesis that an intracellular element is responsible for the disease. More than eighty years later (1953), Francis Crick and James Watson deciphered the structure of DNA (deoxyribonucleic acid) (figure 1-01) and postulated the structure offered a copy mechanism for genetic material.[2]

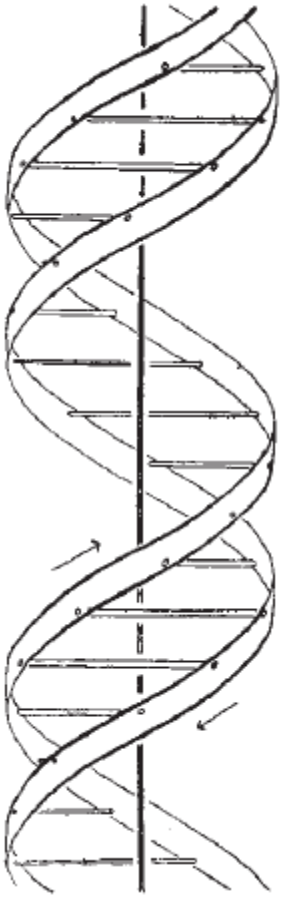


Figure 1-01: The structure of DNA as proposed by James Watson and Francis Crick. Reprinted by permission from Macmillan Publishers Ltd: Nature, copyright 1947.[2]

Following the understanding of DNA structure and DNA as genetic material at the molecular level, astonishing advancements have been made toward understanding the fundamental aspects of cancer at the molecular level. The new found knowledge led to the war against the cancer and to the development of anticancer therapies. Cancer is a genetic disease involving a multistep process directed by the amassing of genetic and epigenetic changes culminating in the uncontrolled growth and spread of abnormal cells. A normal healthy body is comprised of trillions of cells coexisting in an intricate interdependent environment. Normal cells duplicate upon instruction from other cells in their vicinity, collaborating to guarantee each

tissue maintains an appropriate size and structure. Cellular collaboration consists in coordinating behavior through sending, receiving, and interpreting a complex set of signals that serve to control the cells' social interactions. In healthy cells, the signals regulate the resting, dividing, differentiating, and death of the cell as required for the good of the organism. Cancer cells and their progeny reproduce in contempt of normal cellular restraints for division forming masses of abnormal cells (tumors) that invade nearby tissues and/ or migrate to distant sites forming masses of abnormal cells (tumors). As the disease progresses, the cancer cells become more aggressive and malignant, eventually disrupting tissues and organs vital to the organism.[3]

At the molecular level, cancer primarily results from successive mutations that collectively alter specific locations in DNA and cause changes in proteins encoded by cancer related genes. Mutations observed in cancer cells comprise a diverse range, including point mutations, deletions, and insertions involving only a few nucleotides to chromosomal aberration encompassing millions of nucleotides. Genes are contained in the DNA molecules of the cell and designate a sequence of amino acids required to make a particular protein which will execute the function of the gene. Mutations in genes can modify expression, function, and regulation of proteins. In cancer related genes, these mutations usually affect the cell's ability to rest, divide, differentiate, or die.

The gene classes most relevant in cancer are proto-oncogenes, tumor-suppressor genes, and other genes that control cell proliferation. Proto-oncogenes promote cellular-growth and tumor-suppressor genes inhibit growth. The genes *BRAF, c-fos or c-erbb3* are examples of proto-oncogenes, and *RB, p53 and APC* are tumor-suppressor genes.[4] These two gene classes are primarily culpable for the unrestrained cellular proliferation in cancer cells. Mutations that activate proto-oncogenes functionality generate carcinogenic oncogenes that drive excessive and

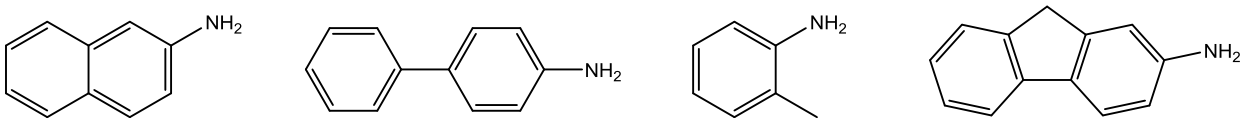
unconstrained cellular division. Mutations that inactivate tumor suppressor genes contribute to cancer through the loss of crucial brakes that prevent uncontrolled growth. Beyond their role in cell proliferation, oncogenes and tumor suppressor genes determine cell fate through differentiation, senescence, and apoptosis.[3] These genes have an essential and irreplaceable role through their regulation in the natural life of cells. The transition of normal cells toward cancer usually involves both mutations activating proto-oncogenes and deactivating tumor suppressor genes. Ultimately it is cooperating genetic defects that result in dysregulation of proliferation, differentiation, and apoptosis that initiate tumorigenesis.[5, 6]

Chemical and Environmental Carcinogenesis

The incidence of cancer has noticeably increased in recent decades in concurrence with increases in unhealthy behavior in the general population, life expectancy, and the presence of carcinogens in consumer products and the environment.[7] Humans are perpetually exposed to exogenous and endogenous chemicals known to induce DNA mutations and cancer. These chemicals are widely referred to as carcinogens. An individual's personal and cultural habits are often the prevailing cause of human cancer. Exposure to carcinogens occurs due to the chemicals presence in food, air, consumer products, or water or the chemical products of cell metabolism. Examples of environmental carcinogens include tobacco smoke, diesel exhaust, asbestos, radiation, and some viruses.[8]

The first evidence of the carcinogenic effects of exogenous chemicals was found in the 18th century. In 1761, Dr. John Hill linked the development of nasal cancer to the excessive use of tobacco snuff.[9] One of the first occupationally related cancers was identified 14 years later by Sir Percival Pott. Sir Percival Pott related increased incidence of scrotal cancer in chimney

sweeps to a continuous exposure to soot.[10] In 1915, Yamagiwa and Ichikawa were the first to experimentally provoke carcinogenesis through repeatedly painting coal tar on the ears of rabbits. The coal tar induced multiple squamous cell carcinomas in the painted areas.[11, 12] The experiments led to identification of a pure carcinogenic chemical, 1,2,5,6-dibenzanthracene a polycyclic aromatic hydrocarbon (PAH) by Ernest Kenneway and his coworkers.[13] Another PAH, pentacyclic benzo[a]pyrene was later identified as a tumorigenic component of coal tar. In an analogous study, 2-naphthylamine (2-NAPH, **1.01**) was shown to induce bladder tumors in canines. Other sources evidenced several simple aromatic amines (SAA) in addition to 2-NAPH were potent carcinogens in animal studies, including 4-aminobiphenyl (4-ABP, **1.02**), o-toluidine (o-tol, **1.03**), and 2-aminofluorene (2-AF, **1.04**) (figure 1-02). Aromatic amines are rather widely used in the aniline dye industry, as antioxidants in the production of rubber and cutting oils, as pesticides and in pharmaceutical chemistry as the initial or intermediate products.[14, 15]



2-naphthylamine (**1.01**) | 4-aminobiphenyl (**1.02**) | o-toluidine (**1.03**) | 2-aminofluorene (**1.04**)

Figure 1-02: Structures of simple aromatic amines.

By the 1940s it had been found that aromatic amines, PAH's and other environmental media were factors in inciting lung, bladder, liver and possibly breast cancer. The results were the first evidence that structurally defined compounds that existed in industry were related to carcinogenesis in humans. At present, a great variety of compounds have been identified as carcinogenic to humans. By the end of 1970's researchers were questioning what the mechanism of action of these carcinogens in genotoxicity. Of particular importance, the work of the Millers and others showed that most carcinogens were metabolized or bio-activated to more chemically

reactive species (metabolite).[16, 17] These species were labeled the "ultimate carcinogens". The "ultimate carcinogen" was initially considered to be an electrophile formed in vivo with a finite stability that could diffuse limited distances to covalently modify DNA. The belief the species would diffuse limited distances led to the idea that carcinogens would be activated in vivo by nuclear enzymes.[18, 19] Years later, it was demonstrated that a carcinogen could be activated to a form capable of covalently modifying DNA with in hepatocytes, and the reactive form could be trapped outside of the cell.[20] In addition, enzymes that are involved in the detoxification of cells were able to activate chemical carcinogens. For example, cytochrome P4501A2 initially activates 4-ABP through N-oxidation generating a reactive species that can covalently modify DNA.[21, 22]

The Role of DNA adducts in Carcinogenesis

As mentioned previously Watson and Crick determined the structure of DNA through X-ray diffraction images. They reported "the novel feature of the structure is the manner in which the two chains are held together by the purine and pyrimidine bases… the (bases) are joined together in pairs, a single base from one chain being hydrogen-bonded to a single base form the other pyrimidine for bonding to occur…Only specific pairs of bases can bond together. These pairs are: adenine (purine) with thymine (pyrimidine), and guanine (purine) with cytosine (pyrimidine)."

As stated in Watson and Crick's report, the outer portion of the DNA double helix is alternating ribose sugar molecules that are linked by phosphate groups. The two strands extend in opposite directions with the nitrogenous bases "inside" the two strands like rungs on a ladder. Each complete "rung in the ladder" consists of a phosphate group, ribose group, and the

nitrogenous base that spans space between the strands, which grouped together are called a nucleotide. Within the DNA double helix structure, each nucleotide of one strand is tightly base-paired with its complementary nucleotide on the opposite strand.[2] The right-handed helix discovered by Watson and Crick is known as B DNA. This is the most common form of DNA found in organisms at neutral pH and physiological salt concentrations. Other forms of DNA reported in organisms are A and Z DNA.[23,24]

As mentioned, DNA holds the genetic information of the organism. The order of the bases in the DNA sequence is the genetic code, where an open reading frame can be transcribed into mRNA and later translated into proteins, required for functioning of living organisms. Each amino acid of a protein is coded buy three bases of a DNA sequence. Cellular division requires proper replication of the genetic code for biological functions to be conserved. Normal replication involves high fidelity polymerases (pol) that strongly favor insertion of correct nucleotide. These are members of the B-family of DNA polymerases, and they include δ, ε, and α. These polymerases misinsert one base for every one million bases that are copied[25]. Several replicative polymerases contain a 3'→5' exonuclease activity, which processes the removal of incorrect nucleotides in the event of misincorporation. However, damaged or modified DNA can halt replication if not repaired. Without repair, accumulated DNA damage leads to mutations. Intricate DNA repair systems maintain genome integrity, in the event that replication errors, environmental insults, and the cumulative effects of age damage the DNA. These repair systems include nucleotide excision repair (NER), base excision repair (BER), and mismatch repair (MMR). Failure of these repair system results in the accumulation of damage.[26] The reaction of carcinogens with DNA is considered one of the earliest events in the initiation of cancer. Many carcinogens react with DNA to form covalent complexes modifying a base in the

sequence. The modified base is referred to as DNA adduct. DNA adducts not repaired prior to replication can cause mutations in the genes involved in important cellular functions as was discussed earlier.

Carcinogens often induce a complex set of mutations thought to be initiated when a DNA polymerase encounters a mutagenic DNA adduct during replication. Adducted DNA may cause mutations through misincorporation or slippage by a DNA polymerase during replicative bypass.[27] Endogenous DNA damage results through insults to DNA by reactive oxygen species (ROS) such as hydroxyl radicals, spontaneous depurinations or depyrimidination, and deamination of the bases adenine, cytosine or guanine. This endogenous damage also contributes to mutagenesis.[28] As discussed earlier, carcinogens are often metabolized to more reactive species that can covalently modify DNA. Carcinogens requiring metabolic activation include polycyclic aromatic hydrocarbons, aromatic amines, nitrosamines, aflatoxins, other mycotoxins, some alkylating agents and estrogens.[29] DNA adduct formation is now an expected attribute of the most potent carcinogens. Therefore, formation of such adducts is the basis for most strategies in molecular epidemiology.[30,31]

Metabolic activation of carcinogens typically results in a transitory electrophilic compound capable of reacting at nucleophilic sites of cellular molecules, more notably DNA. A variety of enzymes are involved in xenobiotic metabolism such that both oxidizing and conjugating enzymes activate carcinogens toward DNA-binding. The list of enzymes include cytochrome P450s, glutathione-*S*-transferases, sulfonyltransferases, and *N*-acetyl-transferases[32]. Once the carcinogen has been activated, the level of adduction to DNA is directly related to carcinogenic potency.[33,34] In addition, the stability of the DNA adducts is affiliated with the

probability of tumorigenesis.[35] However, there is yet no simple answer for the relevance of DNA adduct formation to assessing cancer risk.

Once DNA adducts form, a complex series of events that leads to cancer. DNA adducts that evade the cellular repair mechanisms may lead to permanent mutations.[36] Failure of a repair pathway to recognize DNA damage will generally result in the stalling of normal genome replicative polymerases, and without an alternative system to process the replication bypass of the damaged adduct apoptosis can occur. The enzymes of this alternative system are translesion synthesis (TLS) polymerases. Translesion synthesis polymerases are responsible for bypass of DNA adducts or lesions that block replication by the general genome replicative polymerases.[37,38] These TLS polymerases are in the Y-family and B-family of polymerases. The human TLS polymerases are pol η, κ, ι, and Rev1 from the Y-family and pol ζ from the B-family. Several other bypass polymerases have been discovered, but their role in TLS is not well defined. The TLS polymerases are notorious for their low fidelity resulting in frequent misincorporation of nucleotides. The low fidelity is usually attributed to the TLS polymerases larger active site compared to replicative polymerases. For example, the Y-family polymerase DPO4 from archaeal bacteria *Sulfolobus solataricus* can accommodate two nucleotides within the active site. In addition, Y-family polymerases also lack the 3'→5' exonuclease activity of replicative polymerases, and the TLS polymerase disassociate after the insertion of only a few nucleotides. While TLS polymerases allow the bypass of DNA lesions that halt replication, the low fidelity of these polymerases results in the misincorporations and other mutagenic events.

Events leading to cancer include spontaneous mutations such as base substitutions and frameshift mutations that accumulate throughout the genome in the absence of repair. The complexity of DNA-adduct derived mutagenesis is related to structural and biological factors.

Structural factors include DNA sequence hotspots such as the *Nar*I sequence, which is a hotspot for frameshift mutations. When a mutation occurs in a critical gene and the genes function is affected, carcinogenesis may result. Proto-oncogenes and tumor suppressor genes which regulate cell proliferation are examples of critical genes.[39]

Heterocyclic Amines

The development of human cancer is strongly associated with exposure to environmental carcinogens. As mentioned earlier, food or diet is a common route of exposure. One class of compounds with the most common route of exposure through diet is heterocyclic aromatic amines (HAA). This class of compounds forms in high temperatures when cooking meat through the Maillard reaction.[40,41] The term "Maillard" reaction describes the reaction between amines and carbonyl compounds, especially reducing sugars, during cooking. The reaction is induced at temperatures above 300 °C. Formation at lower temperatures only occurs in the cooking of meats with higher concentrations of amino acids and sugars. Incidentally, the Maillard reaction is of major significance for the development of flavors, texture and brown pigments generated when heating certain foods, and also contributes to the palatability of that food.[41]

Mutagenicity of Heterocyclic Aromatic Amines

The relevance of HAAs was revealed in the early 1970s. Inspired by a study showing mutagens in cigarette smoke, Takashi Sugimura found that particles of smoke, produced by cooking proteinaceous food contained significant quantities of mutagens.[42,43] The mutagens in fumes of frying meat or fish was shown using the Ames *Salmonella typhimurium* test. The Ames test is an easy and sensitive assay to detect potentially genotoxic carcinogens by ascertaining their

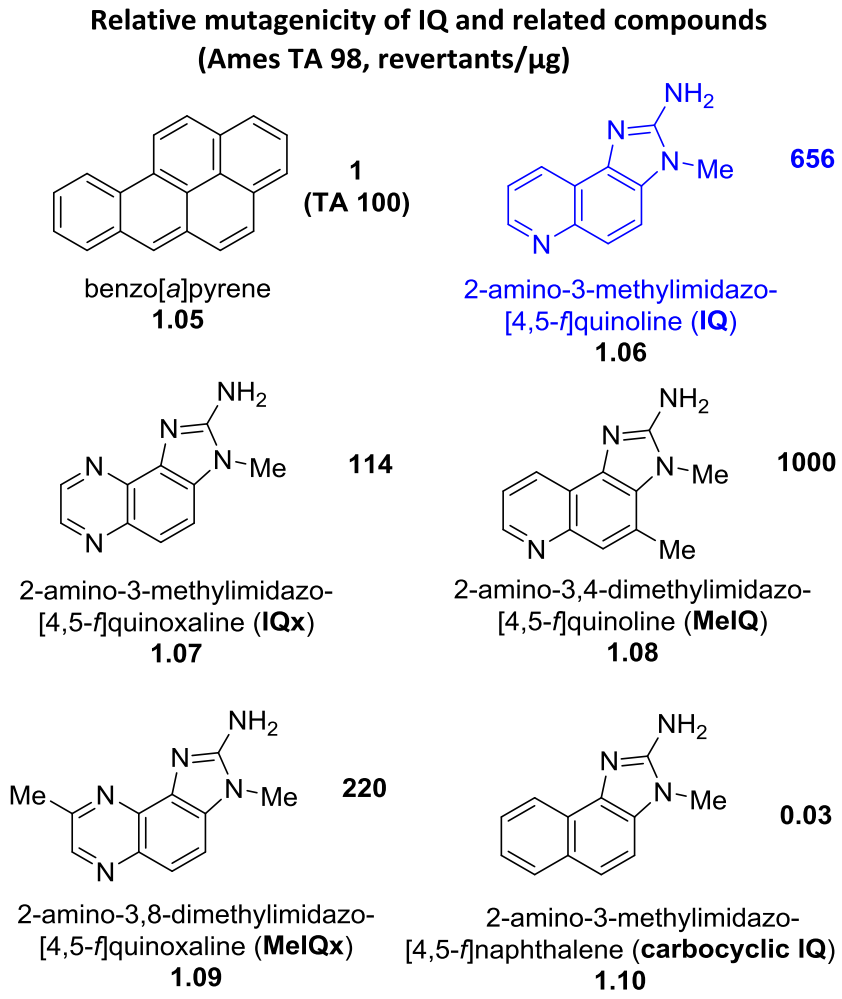


Figure 1-03: Mutagenicity of several HAAs in the Ames TA98 assay

mutagenicity in a bacterial system.[44] The discovery of HAAs had considerable importance in the scientific community. These compounds are isolated from food prepared under normal cooking conditions, and therefore humans are continually exposed. The HAAs are highly mutagenic in the Ames test (Figure 1-03). More than twenty HAAs have been identified to date (Figure 1-04).[43] As a class of compounds, HAAs have also proven mutagenic in mammalian studies in vitro and in vivo. Inflammation, smoking, and diet are responsible for 25-30% of cancers.[45,46] Human exposure to HAAs is modest, one study estimates exposure at ~60 ng/day.[47] Though the levels of exposure are low, HAAs are still thought to contribute to the etiology of human

cancer.[48] They are believed to associated with pancreatic, colon, breast and prostate cancers.[49,50,51,52] The International Agency for Research on Cancer (IARC) have classified many HAAs as either 2A (probable human carcinogens) or 2B (possible human carcinogens) agents.

More than Twenty years of studies evaluating HAA formation in meats have resulted in several methods to reduce HAA intake. Suggested methods to reduce HAA intake include cooking with marinades rich in polyphenols[51,53,54] and not cooking meat over an open flame,[55] flipping meat often[56] or microwaving[57] have all been shown to reduce the formation of HAAs.

2-Amino-3-methylimidazo-[4,5-f]quinoline (IQ)

Of the characterized HAAs, 2-amino-3-methylimidazo-[4,5-f]quinoline (IQ) (**1.06)** is one of the most mutagenic compounds. The International Agency for Research on Cancer (IARC) classified IQ (**1.06**) as a "class 2A" toxin, meaning that IQ is probably carcinogenic to humans. Class 2A is the highest classification of any HAA.[58] IQ (**1.06**) is found in cooked meats at parts per billion (ppb) levels.[59,60] IQ (**1.06**) is also present in tobacco smoke.[61] In Ames' *Salmonella typhimurium* assay, IQ (**1.06**) is a potent inducer of two-base deletions in the CpG dinucleotide repeat sequences of the HisD3052 target sequence (5′ -CGCGCGCG-3′), as are many other aromatic amines and nitrosoaromatic compounds.[44] Studies in which laboratory animal were exposure to IQ (**1.06**) have indicated that IQ is an animal carcinogen. For instance, rats exposed to IQ (**1.06**) through intragastric intubation were found to form tumors in mammary glands, liver and ear ducts.[62] Cynomolgus monkeys also exhibited high levels of hepatocellular carcinomas when IQ (**1.06**) was administered by gavage directly into the stomach.[63] IQ (**1.06**) is primarily metabolized by cytochrome P450 1A2, with cytochromes P450 1A1, 1B1, and 3A4 playing a minor role in the metabolism.[64] Cytochrome P450 1A2 oxidizes IQ (**1.06**) to the *N*-

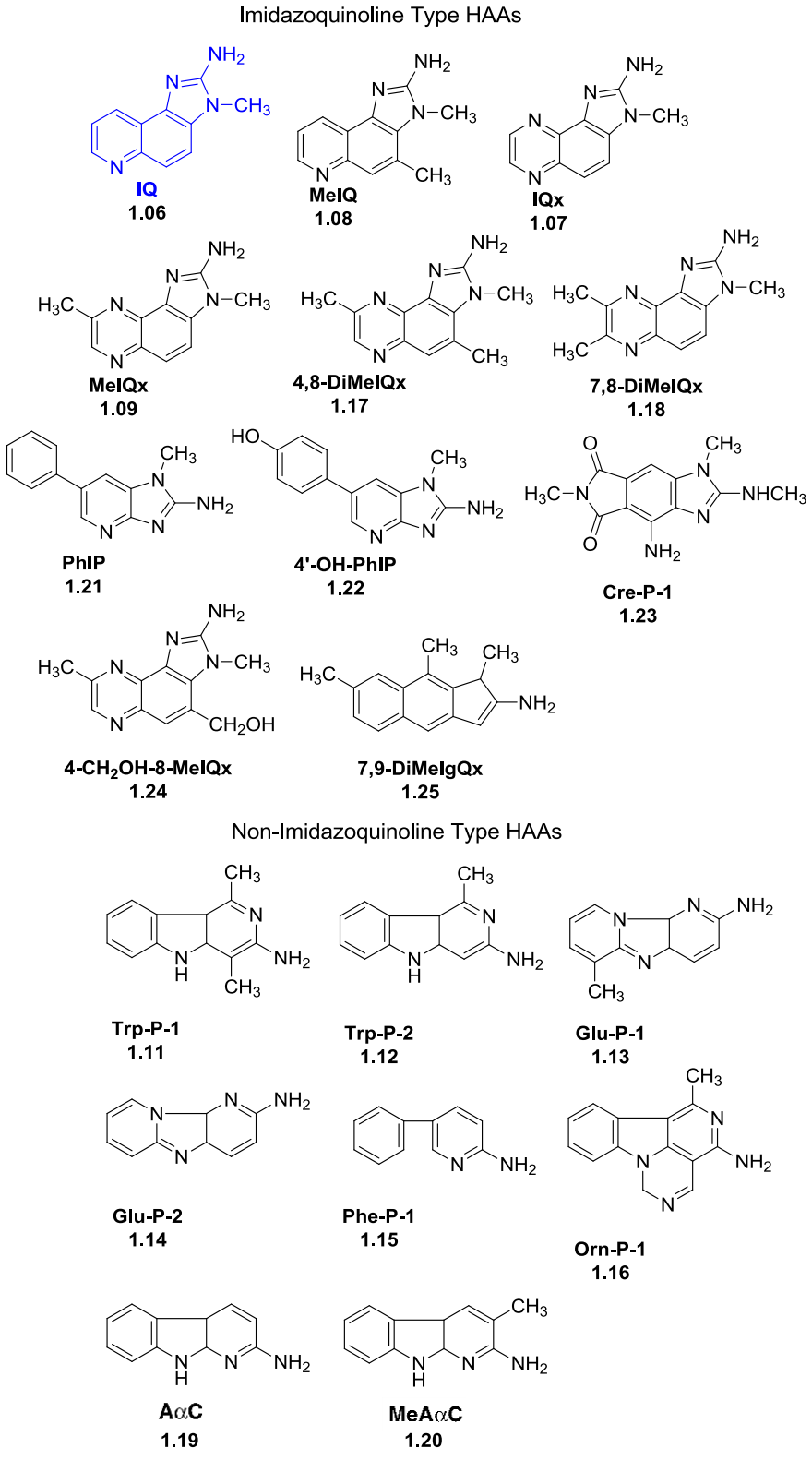
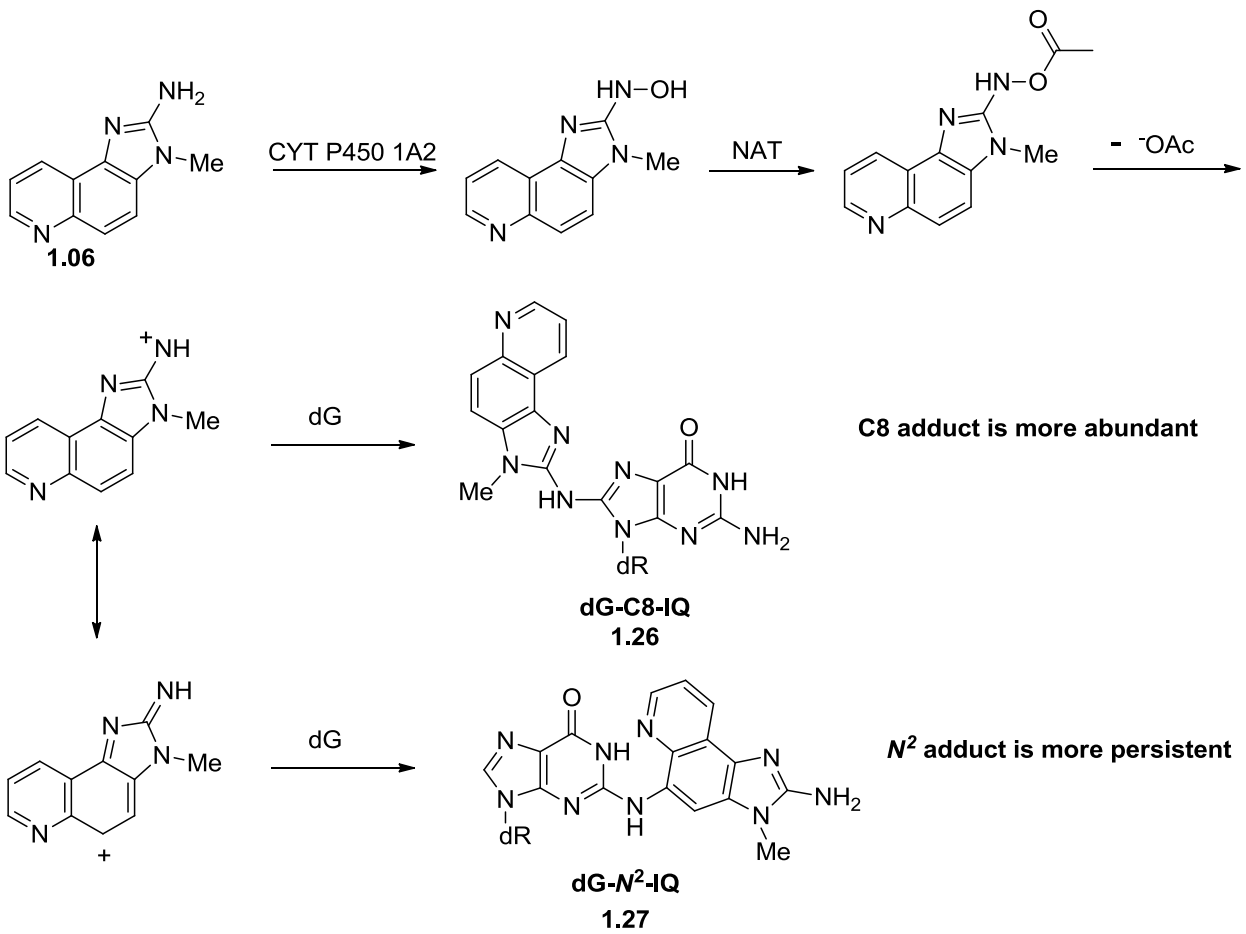


Figure 1-04: 19 HAAs Characterized to Date

hydroxylamine (Scheme 1-01). The *N*-hydroxylamine is then acetylated by N-acetyltransferase (NAT1 and NAT2), particularly NAT2, generating *N*-acetoxy-IQ. Solvolysis may follow acetylation yielding an aryl nitrenium ion. Either N-acetoxy-IQ or the aryl nitrenium ion act as the "ultimate carcinogen" that covalently modifies DNA.64 At the N-hydroxylamine stage,the reaction can continue in the absence of transferase enzymes under acidic conditions.[65] The hydroxylamine may become protonated, and the loss of water through solvolysis will result in the reactive aryl nitrenium ion. However, the metabolism is much more efficient when catalyzed by NAT enzyme. Therefore, tissues with NAT2 activity are more susceptible to formation of IQ (**1.06**) adducts. The major DNA adducts formed from most aryl nitrenium ions occur at the C8

Scheme 1-01: The metabolic activation of IQ



atom of 2′-deoxyguanosine (dG), and a minor product forms at the N^2-posistion of dG. The dG-N^2-IQ (**1.27**) adduct was shown to be more persistent in rodents due to slower repair.[66] The formation of these adducts may cause coding errors at the time of replication. As mentioned earlier, there are repair mechanisms (BER, NER, and MMR) that may repair the damage of dG-IQ adducts. In the absence of repair, the dG-IQ adduct could lead to point mutations, deletions, insertions, or gross chromosomal aberrations.[67]

*Nar*I Recognition Sequence

The *Nar*I recognition sequence is a DNA sequence frequently used to understand the mutagenicity of aromatic amines.[68, 69, 70] The *Nar*I sequence, 5′-$G_1G_2CG_3CC$-3′, contains a GpC dinucleotide repeat. This sequence has exhibited sequence dependent mutagenic properties in *Eschericia coli* (*E. coli*).[71] Repeat sequences are known to produce frameshift mutations in much higher frequencies than non-repeat sequences.[72, 73, 74] The *Nar*I gene codes for the cytochrome b (γ subunit) of a nitrate reductase enzyme that allows the *E. coli* to use nitrate as an electron acceptor during anaerobic respiration.[75] The *Nar*I recognition sequence is a hotspot for frameshift mutations. This site has displayed a mutagenic frequency of 10^7 in *E. coli* over background mutagenicity when G_3 is modified by the aryl amine *N*-acetyl-2-aminofluorene (AAF) (**1.28**) of dG.[76] When the frameshift mutation occurs in *E. coli*, binding of the *Nar*I enzyme to the membrane may be reduced. The binding of the heme may also be reduced, since heme binding is associated with the γ subunit.[77] Streisinger predicted that repeat sequences would be hotspots for mutations.[78, 79, 80] The hypothesis was that either the template or the daughter strand would be able to slip, producing a misalignment of the duplex with bases pushed out of pairing. This would ultimately yield a frameshift mutation.[78] The Ames tester strain TA98

contains an island of $(CpG)_4$ in the HisD3052 target sequence (5′-CGCGCGCG-3′).[44] This is one example of the sequence dependence of the DNA adduct mutagenicity. As mentioned, the *Nar*I restriction sequence 5′-$CG_1G_2CG_3CC$-3′ is a notable mutational hot spot for frameshift mutations for the C8-dG adduct of *N*-acetyl-2-aminofluorene in *E. coli*, especially when the adduct is located at G_3. However, the *N*-acetyl-2-aminofluorene adduct in the *Nar*I sequence induces only base substitutions in simian kidney cells.[81,82,83]

TLS Polymerases η, ι, Rev1, κ, and ζ

Bulky DNA adducts such as dG-C8-IQ (**1.26**) and dG-N^2-IQ (**1.27**) block the genome replicative DNA pols. In this case, replication of the genome can be rescued by the specialized TLS DNA pols than can bypass bulky lesions.[84,85,86,87] These TLS pols are actually more error-prone even with undamaged templates. However, some TLS polymerases can bypass specific DNA damages efficiently and with high fidelity.[88, 89] In eukaryotic cells, efficient TLS is carried out conjointly by two sequential steps.[90, 91] First, a TLS pol replaces the stalled replicative pol and inserts a nucleotide opposite the DNA lesion. Subsequently, the same TLS pol may either extend the primer a few nucleotides beyond the lesion, or it may be replaced by another TLS pol. In either case, the primer is extended a few nucleotides beyond the lesion site before the pol is replaced by the replicative pol to continue DNA synthesis. Studies have found a requirement of accessory proteins for efficient TLS.[92,93] In eukaryotic cells, TLS is carried out by pol η, pol ι, pol κ and Rev1 of the Y-family pols and pol ζ of the B-family enzymes.[87, 94]

Pol η and other Y-family polymerases are less inhibited by the geometric distortions imposed by the presence of lesions in DNA. This allows pol η to replicate through DNA lesions, which typically stall the polymerases typically responsible for duplicating the genome. The

ability of TLS polymerases to better tolerate the geometric distortion of a damaged base and/or a mismatched base pair in their active sites has been substantiated from steady-state kinetic analyses with yeast and human Pol η, which shows the misincorporation of nucleotides with a frequency of $\sim 10^{-2}$ to 10^{-3}.[37, 95, 96] Human pol η stands apart from other TLS polymerase through the ability to replicate past cis-syn deoxythymidine-deoxythymidine (dTyd-dTyd) dimer by inserting two dATP's opposite the dimer with similar efficiency and fidelity as opposite two undamaged dTyd's.[95] Defects in the gene coding of the human pol η cause the cancer predisposition disorder, *xeroderma pigmentosum* variant (XPV).[97,98] Human pol η also plays an important role in the efficient and accurate replication through the 8-oxoguanine (8-oxo-dG) lesion, where other TLS polymerase favor misinsertion of dA opposite 8-oxo-dG.[99,100] However, a low but significant level of dATP is also inserted by pol η. In addition, abasic sites are strong blocks to replication by pol η.[101] It is noteworthy that pol η is inhibited by numerous lesions which severely impinge on the minor groove and/or disrupt Watson-Crick hydrogen bonding of base pairs. Examples of these adducts include N^2-dG adducts of benzo[*a*]pyrene (**1.05**), 7,8-diol 9,10-epoxide (BPDE)[102] and butadiene epoxide;[103] also, the acrolein-derived adduct γ -hydroxy-1,N^2-propano-deoxyguanosine (γ-HOPdG)[104] and 1,N^6-etheno-deoxyadenosine.[105]

Replicative pols as well as TLS pol η and κ form the four possible correct base pairs with nearly equivalent catalytic efficiencies, meaning that dCTP is inserted to form GC base pair as efficiently as dTTP is inserted to form AT base pair. Pol ι is a notable exceptions to this rule.[37] Pol ι incorporates nucleotides opposite the four template bases with very different efficiencies and fidelities. Specifically, pol ι incorporates nucleotides opposite template purines with a much higher efficiency and fidelity than opposite template pyrimidines.[106, 107, 108, 109, 110] Pol ι exhibits the highest efficiency and fidelity opposite template dA. The misincorporation frequencies for

each of the template bases are as follows: opposite A ~10^{-3} to 10^{-5}, opposite dG ~10^{-1}, opposite dC ~10^{-1} to 10^{-2}, and opposite dT misincorporation of dG is 10 fold greater than dA, and dT is inserted as efficiently as dA.[106, 109, 110] For most mispairs, pol ι extends from the mispaired primer terminus with efficiency similar to formation of the mispair. However, it extends the primer-template G:T and T:G mispairs as efficiently as extension from the correct A:T and C:G pairs.[111] Unlike pol η, pol ι is able to incorporate nucleotides opposite an abasic site[106]. A more specific role of pol ι in lesion bypass has emerged from evidence of its proficient ability to incorporate nucleotides opposite of an N^2-adducted guanine.[110] The N^2 group of guanine can conjugate with a variety of endogenously formed adducts. In contrast to replicative/TLS polymerases, pol ι synthesizes DNA using Hoogsteen base pairing, where the primer terminus base is in the syn conformation.[112]

The TLS polymerase Rev1 is more accurately referred to as a deoxycytidyl transferase due to preferential insertion of dCTP.[37, 113] Rev1 typically functions in conjunction with B-family TLS Pol ζ , which is comprised of the Rev3 and Rev7 proteins. For example mutagenesis induced by UV lesions [114, 115, 116, 117] or from abasic sites,[118] requires all three proteins. Rev1 misincorporates nucleotides opposite the template with a frequency of ~10^{-3} to 10^{-4}, and also misinserts a dCTP opposite templates T, A, and C with frequencies of ~10^{-2} to 10^{-3}.[119] Rev1 is necessary for most base substitution mutations induced by UV light,[114] and is indispensable for mutagenesis resulting from TLS through abasic sites.[120] However, the role of Rev1 with these lesions is not C incorporation.[120, 121, 122] In addition, AAF bound to the C8 of a dG presents a strong block to nucleotide incorporation by Rev1. However, studies have implicated a requirement for the Rev1 protein to replicate through the AAF adduct located in a double-stranded plasmid without use of Rev1 of dCTP transferase activity.[123] The implication of these

studies is that Rev1 has a role as an accessory subunit in the TLS of several lesions. Studies indicate the role of Rev1 as an accessory subunit is typically closely associated with assembly of pol ζ.[123, 124] Biochemical studies indicate Rev1 proficiently inserts dCTP opposite γ-HOPdG.[125] Rev1 can proficiently incorporate a dC opposite the various N^2-adducted dG adducts.[125, 126, 127, 128] Structural studies elucidating Rev1 mechanism of action suggest Rev1 dCTP transferase activity will show specificity toward insertion opposite of N^2-adducted dG adducts.[129] Rev1 acts by swinging the template adducted G out of duplex at ~90^0 and forming 2 H bonds on Hoogsteen edge between N^7 and $O^{6^\wedge}$ and Met^{685} and Gly^{686}.[129] The exclusion the template G from the DNA places the N^2 of G in a large (solvent-filled) void between PAD and fingers domain, where an adduct such as γ-HOPdG would be sterically unhindered.[129] In addition to the suggested role of Rev1 in the assembly of pol ζ, more recent studies implicate mouse and human Rev1 in the assembly of pol η, pol ι, and pol κ.[119, 130, 131, 132, 133]

Pol κ belongs to the DinB subfamily of Y-family DNA polymerases including *E. coli* DinB (Pol IV), archaeal Dbh, and Dpo4 proteins.[37, 130] Pol κ is distinct among Y-family polymerases for its nucleotide incorporation specificity and mismatch extension ability. Pol κ is considered the most faithful of all Y-family DNA polymerases with a misincorporation frequency of $\sim 10^{-3}$ to 10^{-4} opposite all four template bases.[37, 134] In contradiction to its fidelity, Pol κ is a proficient extender of mispaired primer termini. Pol κ extension frequency from a mispaired termini is 10^{-1} to 10^{-2}.[37, 135] Several studies support that pol κ is specialized in performing error-free bypass of bulky minor groove N^2-deoxyguanine adducts among other lesions.[136] Investigations also support that pol κ may have a critical role in limiting mutagenesis from specific bulky lesions such as benzo[*a*]pyrene (**1.05**) adducts.[135, 136, 137, 138, 139, 140, 141, 142, 143, 144]

DNA polymerase ζ is a TLS DNA pol sharing sequence similarities within the catalytic domain of its fellow the B family of DNA pols pol δ, pol ε and pol α.[136, 145, 146, 147, 148, 149] Pol ζ consists of both Rev7 and Rev3 proteins.[136, 150] Pol ζ possesses a lower processivity and is devoid of the 3′ → 5′ proofreading exonuclease activity present in most B-family DNA pols. Rev3 alone is capable of polymerization; however, association of Rev3 with Rev7 forms a more stable complex and significantly enhances the polymerase activity of Rev3 by 20–30 fold. This suggests that Rev7 functions as a processivity factor for pol ζ. Recently, the two accessory subunits (pol 31 and pol 32), which are known to associate with the catalytic subunit (pol 3) of yeast pol δ, were also identified in a complex with pol ζ. Furthermore, the presence of pol 31 and pol 32 with pol ζ enhances the efficiency of TLS in yeast.[136, 151, 152] Human analogs of pol 31 and pol 32 identified as pol D2 and pol D3 have been isolated in complex with human pol ζ, and the full complex exhibited polymerase activity in vitro.[136, 153] Thus, pol ζ is now considered to be a four subunit polymerase.[136] Compared to the other Y-family pols, pol ζ is a high-fidelity enzyme, since it misincorporates nucleotides opposite all four template bases with the same frequency as pol α, $\sim 10^{-4}$.[37, 106, 120, 154] Pol ζ differs significantly from other DNA pols in its very proficient ability to extend from mispaired primer termini, which occurs with a frequency of $\sim 10^{-1}$ to 10^{-2}. Pol ζ is very inefficient in replicating through most DNA lesions, which results from its inability to incorporate nucleotides opposite them.[37, 136] Pol ζ is particularly specialized to extend distorted base pairs, such as mismatches that may result from inaccurate base insertion or a base pair involving a bulky DNA lesion.[155, 156] Thus, the primary role of pol ζ in TLS is performing extension following insertion opposite of the lesion by another TLS pol.[157]

Dissertation Aims

As previously discussed, carcinogens can induce mutations. These mutations are thought to be initiated at the time of replication when a DNA polymerase encounters a carcinogen-modified nucleobase. Misincorporation of the wrong base by a DNA polymerase results in a mutation in the sequence, and an accumulation of persistent mutations and other DNA damage will ultimately lead to cancer.

The aim of this dissertation is to examine the in vitro replication of the dG-C8-IQ (**1.26**) and dG-N^2-IQ (**1.27**) adducts by translesion DNA polymerases to determine their contributions to the observed mutagenic spectra. In this case, IQ adducts are known to induce G→A transitions and G→T transversions in eukaryotic cells.

Chapter II

In Chapter II, the in vitro replication of the dG-C8-IQ (**1.26**) adduct is examined by TLS polymerases in relation to the mutagenic profile observed through small interfering ribonucleic acid (siRNA) knockdowns of TLS polymerases in eukaryotic cells. This allowed elucidation of the polymerases involved in the mutations initiated by translesion bypass of the dG-C8-IQ (**1.26**) adduct. The aims of Chapter II are:

1. Discussion of the mutagenic profile observed for dG-C8-IQ (**1.26**) through siRNA knockdowns of TLS polymerases in HEK293T (human embryonic) kidney cells.
2. Examination of the previously unreported translesion bypass of dG-C8-IQ (**1.26**) by yeast DNA polymerase ζ and the deoxycytidine triphosphate (dCTP) transferase human Rev1.
3. Examination of the extension from a mismatched primer termini, dG-C8-IQ:N where N is A or T by TLS polymerases ζ, κ, ι, and η.

Chapter III

In Chapter III, similar to Chapter II, the in vitro replication of the dG-N^2-IQ (**1.27**) adduct is examined by TLS polymerases. The in vitro replication results are related to the mutagenic profile observed through siRNA knockdowns of TLS polymerases in HEK293T kidney cells. This allowed elucidation of the TLS polymerases involved in the mutations initiated by translesion bypass of the dG-N^2-IQ (**1.27**) adduct. The aims of Chapter III are:

1. Discussion of the mutagenic profile observed for dG-N^2-IQ (**1.27**) through siRNA knockdowns of TLS polymerases in HEK293T kidney cells.
2. Examination of the previously unreported translesion bypass of dG-N^2-IQ (**1.27**) by yeast DNA polymerase ζ and the dCTP transferase human Rev1.
3. Examination of the extension from a mismatched primer termini, dG-N^2-IQ:N where N is A or T by TLS polymerases ζ, κ, ι, and η.

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CHAPTER II

REPLICATION OF THE C8-DEOXYGUANOSINE IQ ADDUCT

Introduction

TLS: Incorporation of Nucleotides Opposite of dG-C8-IQ

dG-C8-IQ (**1.26**) is the major adduct formed with dG in DNA and the bio-activated form of the probable carcinogen IQ.[1, 2, 3] Previously the *in vitro* replication of the dG-IQ adducts has been investigated. The IQ lesions were site specifically incorporated into 27mer templates at the G_1 and G_3 positions of the *Nar*I recognition site.[4] As discussed in Chapter 1, sequences with GC dinucleotide repeats are susceptible to frameshift mutations. As a result, these sequences are of interest to determine if a particular DNA adducts is capable of inducing frameshift mutations. For example, the replication of the dG-AAF adduct especially when located at the iterated G_3 positon (5′-$G_1G_2CG_3$CC-3′) is susceptible to frameshift mutations in *E. coli.*[5, 6, 7] However, dG-AAF induces only base substitutions in simian kidney cells.[5, 6, 7] The previous studies revealed the ability of TLS pols η, κ, ι and the B-family replicative pol δ to incorporate nucleotides opposite of and past both the dG-C8-IQ (**1.26**) and dG-N^2-IQ (**1.27**) lesions. Replication by pol δ is completely blocked by the IQ adducts. When present in the *Nar*I restriction site, human pol η extended primers beyond dG-C8-IQ (**1.26**) more efficiently than pol κ and much better than pol ι.[4] TLS by pol η was determined to be largely error-free for the dG-C8-IQ (**1.26**) lesion. Pol η inserted dCTP and dATP opposite of the dG-C8-IQ (**1.26**) lesion, and the misinsertion frequency of dATP by pol η was .0097 at G_1 and 0.43 at G_3. However, no extension was observed when pol η inserted dATP opposite of the lesion.[4] Human pol κ was capable of inserting dCTP and dGTP

opposite of the dG-C8-IQ (**1.26**) adduct, and then further extension was blocked.[4] The misinsertion frequency of dGTP by pol κ was .047 at G_1 and .069 at G_3. The same study revealed human pol ι would insert dCTP or dTTP opposite of dG-C8-IQ (**1.26**) lesion.[4] Pol ι was incapable of further extension following insertion, and the reported misinsertion frequency of dTTP was 2.0 at G_1 and 5.8 at G_3.[4] The misincorporation results of this *in vitro* study suggested that TLS replication of dG-C8-IQ (**1.26**) could result in GC→AT transitions, or the transversions GC→TA, GC→CG.[4] Further studies in HEK93T cells would reveal that the observed mutations resulting from replication of dG-C8-IQ (**1.26**) were the point mutations, G→A and G→T.[8] Thus, the *in vitro* insertion of dGTP by pol κ was irrelevant to the mutagenesis induced by the lesion.

Induced Mutagenesis siRNA knockdown of TLS Polymerases

The mutagenesis of the dG-C8-IQ (**1.26**) adduct in HEK293T (human embryonic) kidney cells has been investigated utilizing siRNA knockdown of the TLS pols.[8] This study specifically investigated the role of various TLS pols in bypassing dG-C8-IQ (**1.26**) located at the three guanine positions of the *Nar*I restriction site in human cells.[8] The complete inability to express genes for TLS polymerases in humans could result in cell death.[9, 10] siRNA knockdown involves partial silencing of a target gene.[11] siRNA knockdown is utilized in the case that the target protein is critical to cell viability. The result of the technique is that the amount of protein is reduced to the point that loss of function yields critical data regarding the role of the protein.[11] In this particular investigation, the siRNA knockdown of the targeted TLS pol(s) resulted in at least 70% silencing. The lesion containing or unmodified (for control) oligonucleotide was ligated in to a plasmid vector. Then, following siRNA knockdown of the target TLS pol (s), the plasmid

was replicated in HEK293T cells. Ultimately, the replication products were recovered. Products not hybridizing to the complementary wild-type 14mer sequence were considered mutants and were subjected to sequence analysis. The negative control, where TLS was not reduced, revealed that dG-C8-IQ (**1.26**) is bypassed more efficiently at $G_3 > G_2 > G_1$ (81% TLS at G_3 vs 58% TLS at G_1) (Figure 2-01). The TLS pols η, κ, ι, ζ and Rev1 were evaluated for bypass of dG-C8-IQ (**1.26**). Each of the TLS pols evaluated were involved in replication of dG-C8-IQ (**1.26**) adduct to some extent as indicated by the reduction in the % TLS relative to wild-type (WT) for each knockdown performed (Figure 2-01). The simultaneous knockdown of pol η/pol ζ showed the most pronounced effect on TLS with up to a 70% reduction in viability of the dG-C8-IQ pol ζ play critical roles in TLS of dG-C8-IQ (**1.26**), although pol κ also is important. The DNA

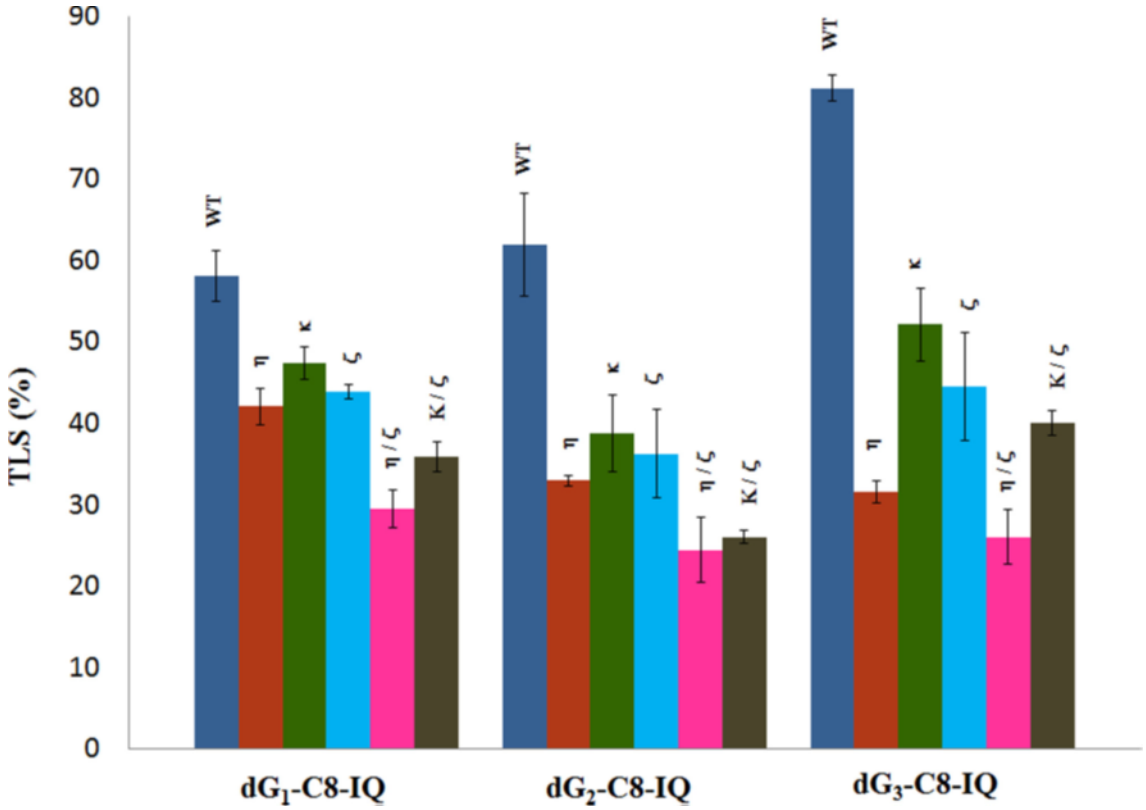

Figure 2-01. The effect of the siRNA knockdowns of TLS polymerases on the percentage of replicative bypass of the dG-C8-IQ. The method by which the percentage of TLS in observed for the depicted knockdowns is described in the corresponding publication.[8]

sequencing analysis revealed that G→T transversions were the main mutations observed, however, some G→A transitions were also seen. Significantly, no frameshift mutations were observed. The analysis also showed that dG-C8-IQ (**1.26**) is mutagenic in HEK293T cells in all three sites (figure 2-02). The order of mutational frequency (MF) for each adducted G was $G_3 > G_2 > G_1$ with fifty percent MF at G_3. Knockdown of pol η resulted in an increase in MF, which was most pronounced at G_3 (26% increase in MF in the progeny from G_3) (Figure 2-02). Therefore, pol η is essential to error free bypass of the dG-C8-IQ (**1.26**) lesion as there are fewer mutations during TLS by active pol η. In contrast, MF at each site was decreased when pol κ, ι, ζ or Rev1 was knocked down (Figure 2-02). Thus, these polymerases are collectively responsible for the error-prone TLS of dG-C8-IQ (**1.26**). The most pronounced decrease in MF at each site was in pol κ-knockdown cells. MF of the progeny derived from the G_1, G_2 and G_3 constructs, respectively, were reduced by 47%, 67% and 38% upon knockdown of pol κ. In addition, the simultaneous knockdown of two pols showed that the lack of each two-pol combination resulted in further decrease in MF indicating a more error prone processivity. For example, a decrease in MF was observed when pols η and κ were simultaneously knocked down (Figures 2-02), where the individual polymerases exhibited opposite effects. In addition, the simultaneous knockdown of pol ζ and Rev1 together, and that of pol κ, ζ, and Rev1 together, decreased MF at each adducted guanine in the sequence much more than anyone pol individually (Figures 2-02). Simultaneous knockdown of pols κ and ζ exhibited a remarkable synergy on the reduction of MF (Figure 2-02). Triple-pol knockdown of pol κ, pol ζ and Rev1 further reduced the MF of the progeny from each dG-C8-IQ (**1.26**) construct. Based on this result, the study concluded that the most critical role in the error-prone TLS of the dG-C8-IQ (**1.26**) adduct is played by pols κ and ζ, whereas pol ι likely has a relatively minor role. The study also concluded that pol η plays a

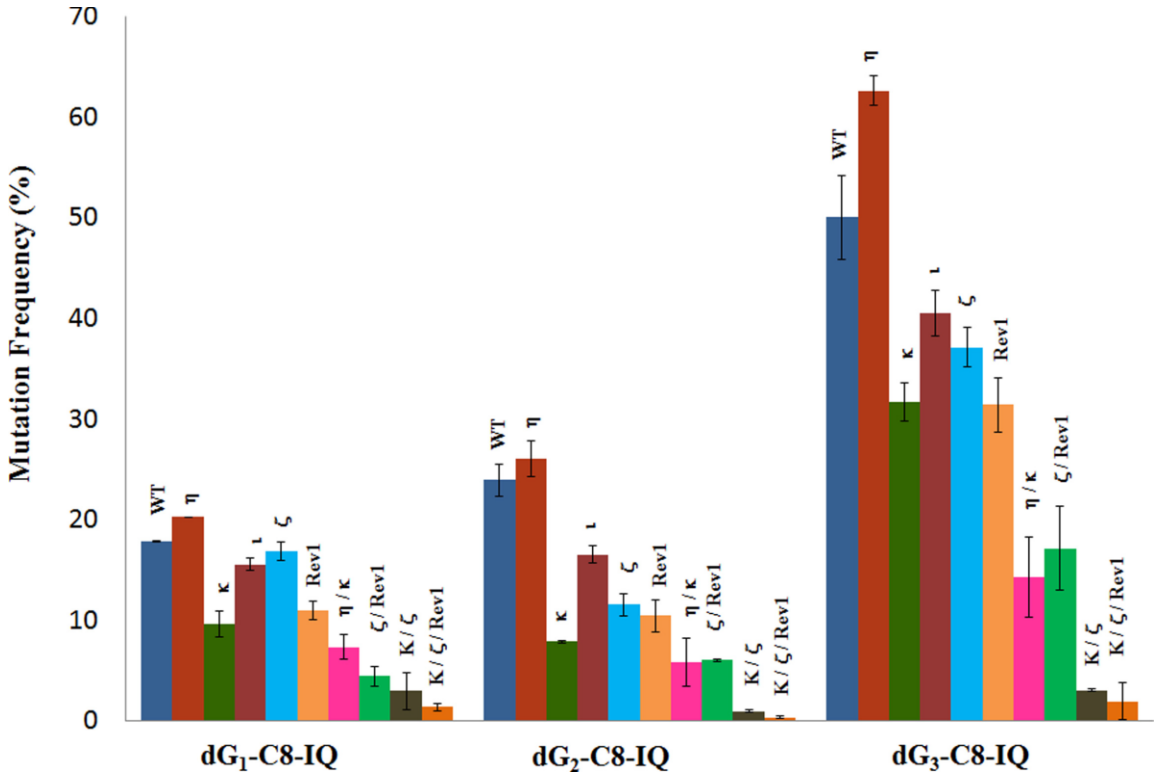

Figure 2-02. The depicted results represent the mutational frequency resulting from TLS of dG-C8-IQ at G_1, G_2 and G_3 of the oligonucleotide constructs in HEK293T cells where the cells were transfected with NC siRNA (WT) or siRNA for single, double or triple pol(s) knockdowns (according to each bar label). Details of the experiment and result interpretation are available in the corresponding publication.[8]

critical role in error free TLS of the dG-C8-IQ (**1.26**) lesion.[8]

The structure of dG-C8-IQ (**1.26**) at each guanine site in the *Nar*I recognition site in the duplex has also been reported. At the G_1 and G_2 positions of the *Nar*I site, dG-C8-IQ (**1.26**) is located in the minor groove and at the G_3 position dG-C8-IQ (**1.26**) is intercalated, and the adduct was found to be in the syn conformation.[12] Overall, Pol η, κ, and ζ are the primary TLS polymerases implicated in the bypass of the dG-C8-IQ (**1.26**) adduct.[8] The siRNA knockdown study also revealed that the primary mutation induced by dG-C8-IQ (**1.26**) in HEK293T cells was G→T transversions, and the minor mutation was G→A transitions.[8] No frameshift mutations and no G→C transversions were induced by dG-C8-IQ (**1.26**) in HEK293T cells.[8] Furthermore, previous *in vitro* study showed that human pol η extended primers beyond dG-C8-IQ (**1.26**) adduct at either G_1 or G_3 more efficiently than pol κ or pol ι (η > κ > ι).[4] This

study also established that pol η is capable of the misinsertion of dATP opposite the dG-C8-IQ (**1.26**) adduct, but could not extend from dG-C8-IQ:A base pair, and that pol ι is capable of the misinsertion of dTTP, but not extension from the dG-C8-IQ:T base pair.[4] Further extension after the misinsertion of dATP by η as demonstrated in the *in vitro* study, would yield the G→T transversion observed in HEK293T cells.[4, 8] Similarly, pol ι is capable of misinserting dTTP, which would yield the G→A transition observed in HEK293T cells upon further extension.[4, 8] However, the *in vitro* study implicates pol η and ι are incapable of extending from the respective mismatch base they insert opposite of the dG-C8-IQ (**1.26**) lesion.[4] The current study investigates the *in vitro* TLS polymerases involved in extending from the mismatched base pairs, dG-C8-IQ:A and dG-C8-IQ:T, that will yield the G→T transversion and the G→A transitions observed in HEK293T cells. We were particularly interested in the potential roles of pols κ and ζ, which were found to be most responsible for mutagenic TLS of dG-C8-IQ (**1.26**).

Results and Discussion

TLS by Pol ζ and Rev1

In the previous bypass study, no *in vitro* TLS experiment using pol ζ or Rev1 were conducted. In the current study, the bypass dG-C8-IQ (**1.26**) at G_1 and G_3 in the *Nar*I restriction site with these specialized pols was evaluated. A 5′-^{32}P-labeled 19mer or 22mer (-1) primer was annealed to appropriate complementary template strand containing the dG-C8-IQ (**1.26**) adduct (0-position) (Figure 2-03). Primer extension assays were performed with pol ζ in the presence of all four deoxyribonucleotide triphosphates (dNTPs) to determine the capability of pol ζ to perform TLS of dG-C8-IQ (**1.26**). The primer extension was performed with Rev1 in the

presence of dCTP. Yeast pol ζ (ypol ζ) and Rev1 (Figure 2-04) were unable to incorporate a nucleotides opposite dG-C8-IQ (**1.26**) at either G_1 or G_3. The lack of nucleotide insertion by ypol

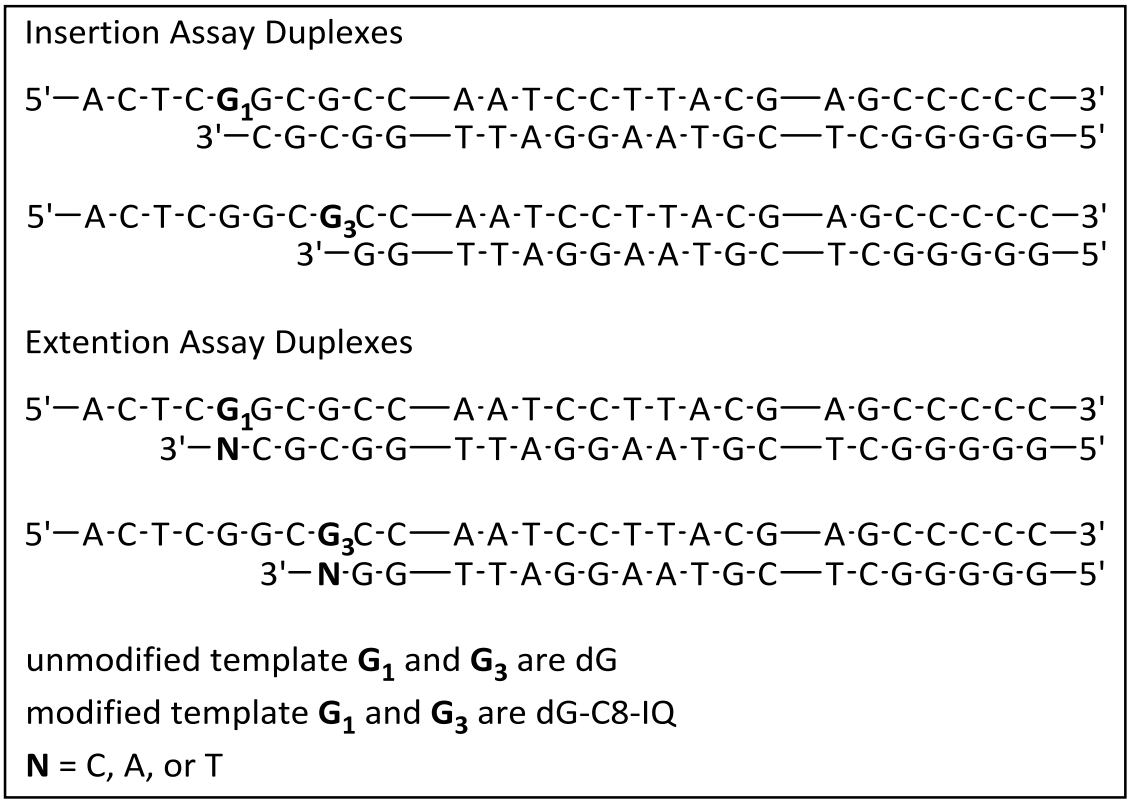

Figure 2-03: Duplexes utilized in TLS assays

ζ and Rev1 opposite of the dG-C8-IQ (**1.26**) adduct is consistent with the reported roles of the polymerases. The primary role of pol ζ in TLS is reportedly performing extension following insertion opposite of the lesion by another TLS polymerase.[13] In addition, Rev1 is reported to proficiently incorporate a dC opposite of various N^2-adducted dGs, otherwise Rev1 acts frequently as an accessory protein.[9, 14] As pol ζ typically acts as an extender, the role of ypol ζ to extend from a dG-C8-IQ:N template-primer junction was evaluated (Figure 2-05). Indeed, ypol ζ was observed to extend from dG-C8-IQ:N base pairs at G_1 and G_3, where N is C, A or T, by at least one nucleotide. The three primers represent the major replication outcomes observed in the cell mutagenesis studies. Interestingly at G_3, further extension of three nucleotides beyond the lesion was ~4-fold more efficient (22% versus 5%) for the dG-C8-IQ:A pair than dG-C8-IQ:C

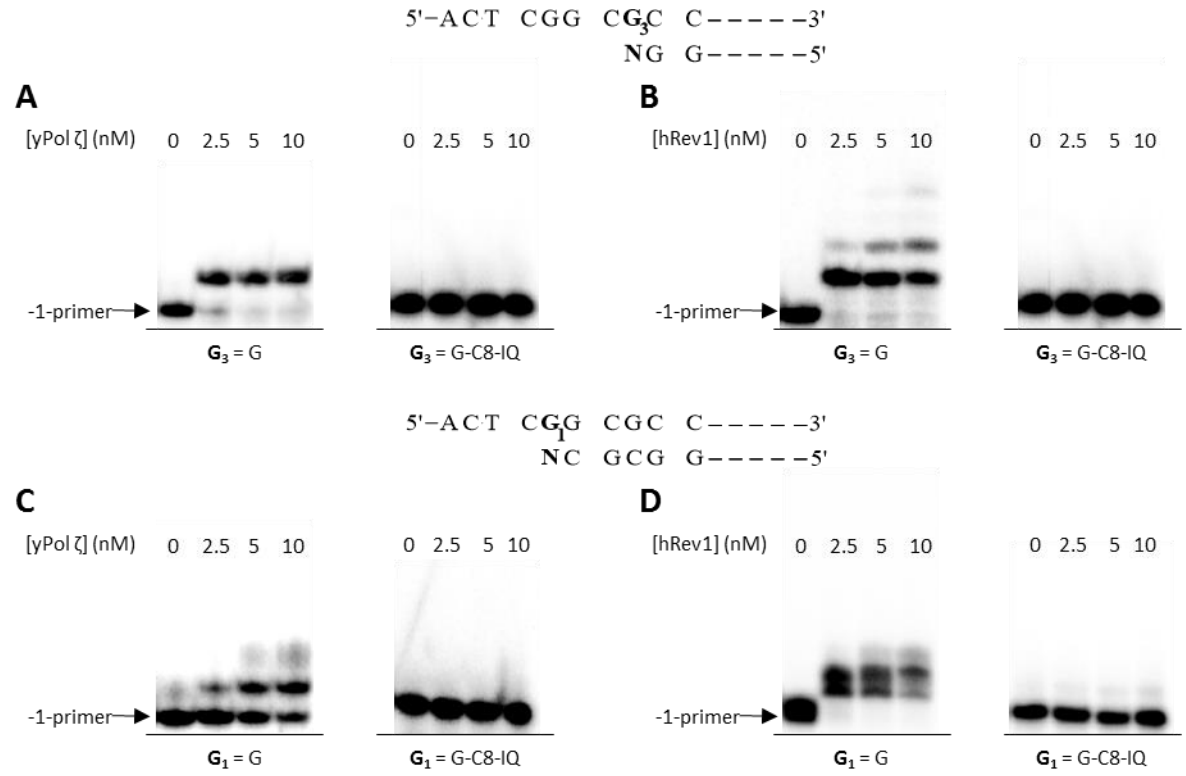

Figure 2-04: In vitro insertion and extension assay of the dG-C8-IQ adduct by ypol ζ and human Rev1 insertion assay. (A) Insertion by pol ζ of dCTP opposite a control unmodified dG (left) and reaction of the dG_3-C8-IQ modified oligonucleotide in the presence of all four dNTPs. (B) Insertion of dCTP (100 μM) opposite a control C (left) or dG_1-C8-IQ (right) by hRev1 at 37 °C. (C) Insertion of dCTP opposite a control unmodified dG (left) and reaction of the dG_1-C8-IQ modified oligonucleotide in the presence of all four dNTPs. (D) Insertion of dCTP (100 μM) opposite a control C (left) or dG_1-C8-IQ (right) by hRev1 at 37 °C. The DNA concentration was 10 nM.

pair, implying pol ζ's critical role in error-prone TLS of dG-C8-IQ (**1.26**) (figure 2-05A). Extension of the dG_3-C8-IQ:T pair by three or more bases was modest (< 2%). At the dG_1-C8-IQ:N primer terminus, where N is C, A or T, ypol ζ does not extend beyond the insertion of one nucleotide (Figure 2-05B). Extension by one nucleotide from dG_1-C8-IQ:C primer terminus was more efficient than for the mismatch pair with A or T. However, ypol ζ is capable of extension from the mispairs, dG_1-C8-IQ:A and dG_1-C8-IQ:T (Figure 2-05B). Extension beyond the one nucleotide inserted by ypol ζ at (Figure 2-05B). Extension beyond the one nucleotide inserted by ypol ζ at the dG_1-C8-IQ:N terminus would require the cooperation of another TLS polymerase. Individually, pol ζ and pol κ are implicated in error-prone TLS of dG-C8-IQ (**1.26**) through the

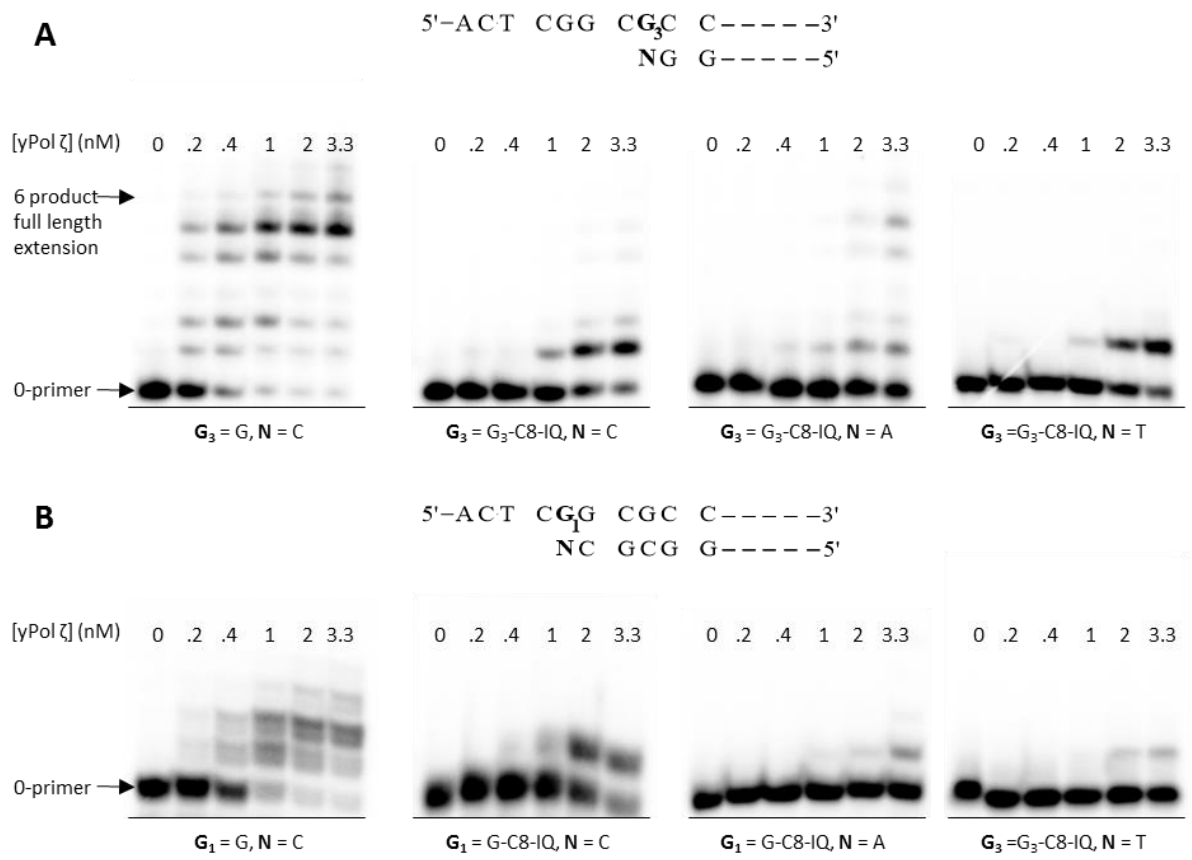

Figure 2-05: *In vitro* extension assay of the dG-C8-IQ adduct by ypol ζ. (A) Extension of the dG_3-C8-IQ adduct when paired with C, A, or T after 5h at 37^0C. (B) Extension of the dG_1-C8-IQ adduct when paired with C, A, or T after 5h at 37^0C. The DNA was 10 nM.

reduction in MF upon siRNA knockdown of the enzyme in cellular studies.[8] The *in vitro* extension from mispairs at G_3 and G_1 with dG-C8-IQ (**1.26**) by ypol ζ is in agreement with the cellular results. However, the incomplete extension of the primer in the *in* vitro assay implies cooperation of an additional polymerase is required. The cellular assays imply pol ζ and pol κ cooperatively perform this error prone TLS as the MF is reduced to less than 5% upon double knockdown of pol ζ and pol κ.[8]

TLS by Pol κ

In the previous *in vitro* study, human pol κ was capable of inserting dCTP or dGTP opposite of the dG-C8-IQ (**1.26**) adduct, but did not extend.[4] Base substitution mutations

resulting from the misinsertion of G are not observed in cellular assays.[8] Therefore, misinsertion does not account for the role of pol κ in error prone TLS indicated by the results of the cellular assays described above. Various in vitro assays with human pol κ are reported here to elucidate the role of pol κ in the error-prone TLS of dG-C8-IQ (**1.26**) at G_1 and G_3 of the of the *Nar*I restriction site. Extension of the dG_3-C8-IQ:N pair was observed for pol κ, where N is A or T but not C, but only at a high protein concentration (Figure 2-06A). Extension of three or more nucleotides past the lesion site was ~8 and 5% for N = A and T, respectively. Interestingly, intermediate extension products for pol κ were in lower abundance than for the ypol ζ extension. This suggests insertion of the first nucleotide past the dG_3-C8-IQ:N pair (+1 position) may be rate limiting for pol κ. Extension of the dG_1-C8-IQ:N pair was observed for pol κ, where N is A or but not C or T, but only at a high protein concentration (figure 2-06B). Extension of two or more nucleotides past the lesion site was ~24% for N = A. The low activity of pol κ in this *in vitro* assay would indicate pol κ has a minor role in error-prone TLS. However, in the cellular assay pol κ contributed more to error-prone TLS than any other individual TLS polymerase evaluated.[8] The cellular studies indicated cooperativity between pol ζ and pol κ. In our *in vitro* results, ypol ζ extended from the mismatch dG-C8-IQ:N, where N is A or T, primer terminus by at least 1 base pair, and the observation was made that further extension would require cooperation of an additional polymerase, most likely pol κ. Thus, the next assay reported examines pol κ's ability to extend a C:G template-primer (+1 primer) terminus containing the dG-C8-IQ:N pair (at the 0-position) at either G_3 or G_1. Pol κ efficiently extended the primer when N was C and A while extension of the primer containing the dG-C8-IQ:T pair was modest at both G_3 and G_1 (figure 2-06C and D). This suggests that pol κ can extend the primer after another TLS pol inserts nucleotides opposite the lesion and its 5′ base. The previous *in vitro*

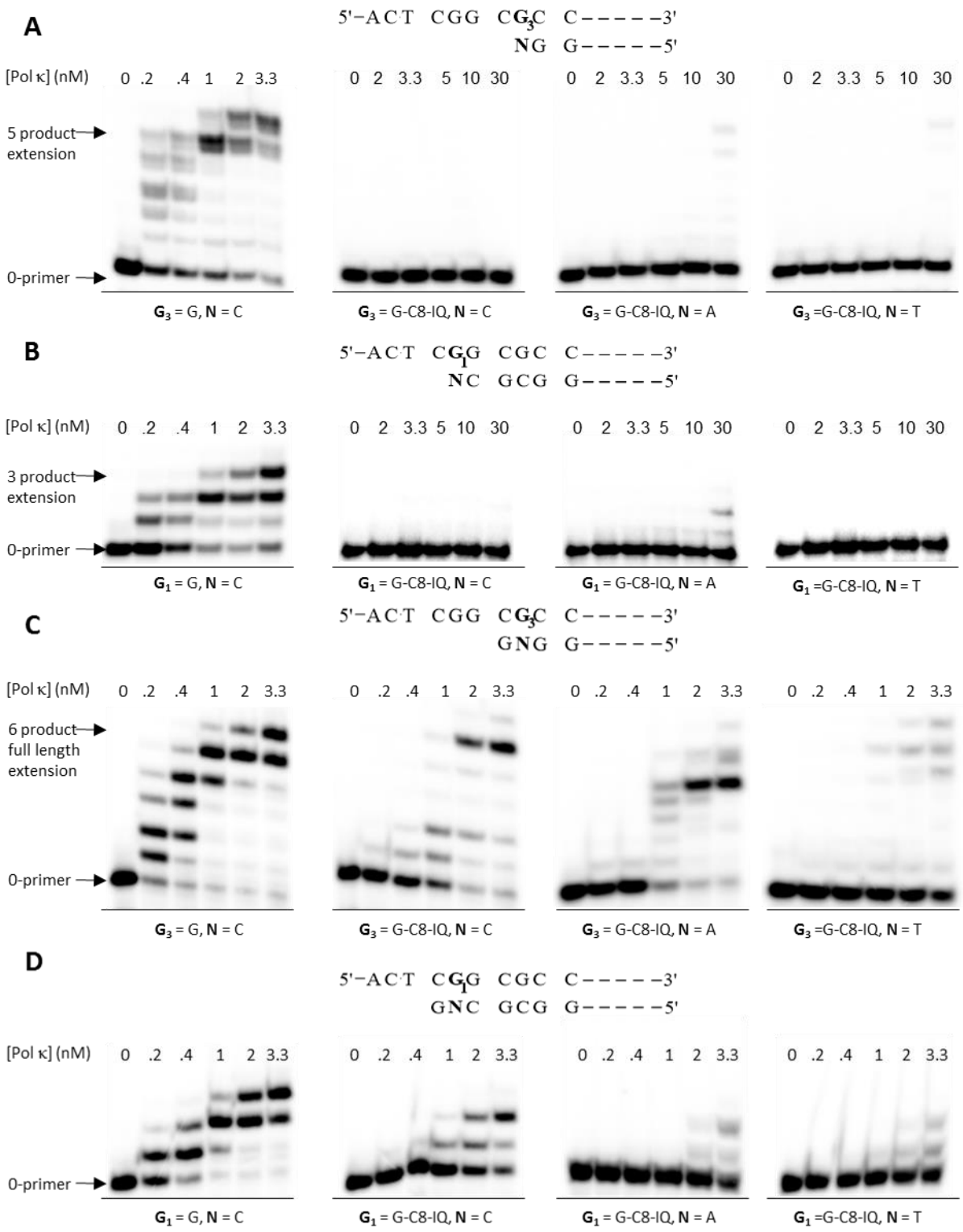

Figure 2-06: (A) Extension past a dG_3-C8-IQ:N pair (N = C, A and T; 10 nM). (B) Extension past a dG_1-C8-IQ:N pair (N = C, A and T; 10 nM) . (C) Primer extension of a G:C primer template terminus (+1 position) and dG_3-C8-IQ:N pair (N = C, A and T; 0-position). (D) Primer extension of a G:C primer template terminus (+1 position) and dG_1-C8-IQ:N pair (N = C, A and T; 0-position). The DNA concentration was 10 nM and was extended by human pol κ after 5 h at 37°C in the presence of all four dNTPs (100 μM).

insertion of the 5′-base from the mispaired, dG-C8-IQ:A or dG-C8-IQ:T, primer terminus and further extension by κ. Pol κ is reported to be a proficient extender of mispaired termini.[9, 15] The above *in vitro* assay indicates that in the case of mispairs with dG-C8-IQ (**1.26**), pol κ requires the cooperation of additional TLS polymerases.

TLS by Pol ι

Cellular studies indicate pol ι plays a minor role in error-prone TLS of dG-C8-IQ (**1.26**) with MF slightly decreasing upon knockdown.[8] In the previous *in vitro* study, human pol ι was less efficient than pol η and κ. Pol ι was capable of inserting C or T opposite of the lesion, then no further extension was observed.4 The previous in vitro study indicates that the misinsertion of

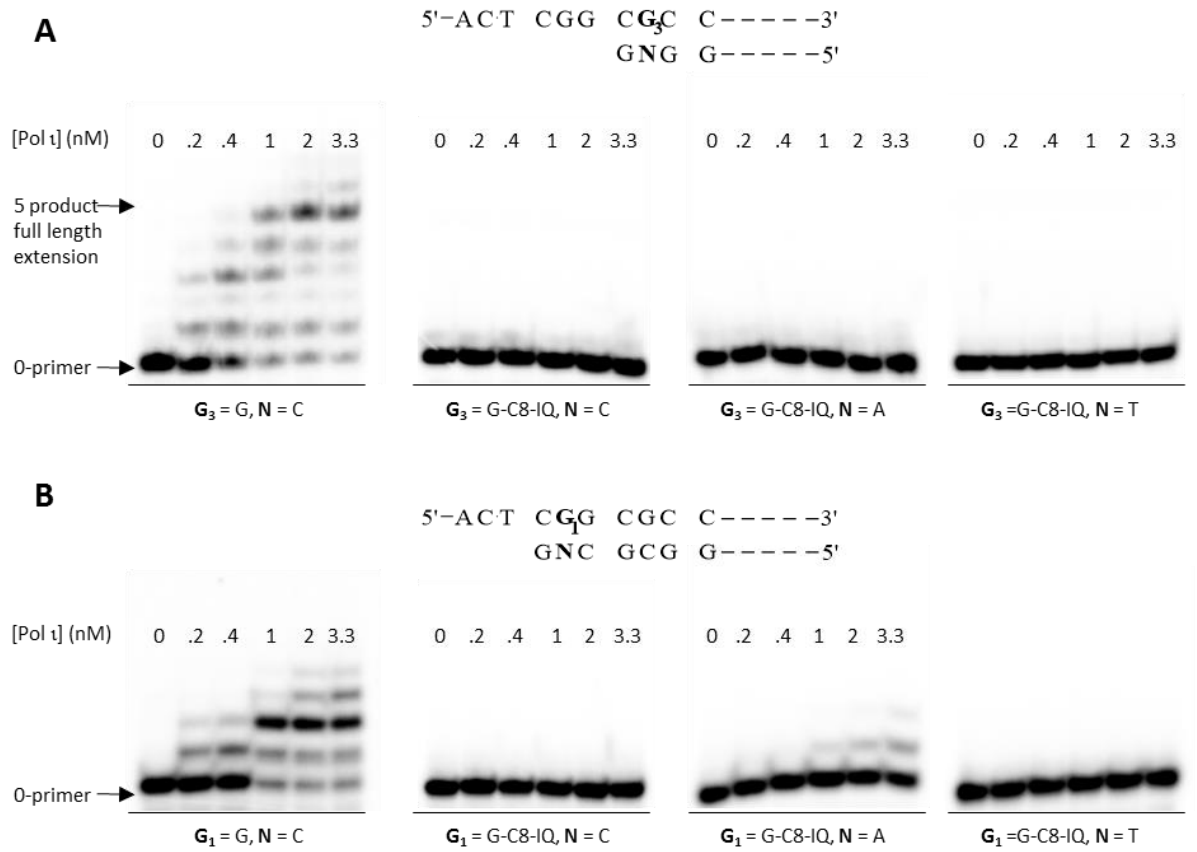

Figure 2-07: *In vitro* extension assay of the dG-C8-IQ adduct by pol ι. (A) Extension of the dG_3-C8-IQ adduct when paired with C, A, or T after 5h at 37^0C. (B) Extension of the dG_1-C8-IQ adduct when paired with C, A, or T after 5h at 37^0C. The DNA was 10 nM.

T is one way pol ι contributes to error-prone TLS. The ability of pol ι to extend in vitro from dG-C8-IQ mismatched primer termini at G1 and G3 of the *Nar*I sequence was evaluated (Figure 2-07). Human pol ι did not extend from the dG3-C8-IQ:N, where N is C, A, or T, primer termini in vitro (Figure 2-07A). In fact, the only extension observed for pol ι was modest extension of the dG1-C8-IQ:A mispair by up to two nucleotides with ~28% extension beyond the zero primer (Figure 2-07B). These results agree with the conclusion of the previous work that pol ι has a minor role in the TLS of the dG-C8-IQ (**1.26**) adduct.4, 8 The differential processing of the dG-C8-IQ (**1.26**) adduct at G_1 vs G_3 of the *Nar*I site may be related to the differences in structure within the duplex, where the adduct is located in the minor groove at G_1 but intercalated at G_3.[12]

TLS by Pol η

Previous *in vitro* studies and cellular studies agree that Pol η is primarily responsible for error-free TLS of dG-C8-IQ (**1.26**) lesions.[4, 8] The siRNA knockdown of pol η increased the MF at guanine in the *Nar*I sequence, including a 26% increase in MF at G_3. The conclusion drawn from the result was that pol η is key in error-free TLS of dG-C8-IQ (**1.26**). However, the results do not preclude pol η from a minor role in error-prone TLS. In fact, pol η was the only enzyme observed to misinsert A opposite of dG-C8-IQ (**1.26**) in the previous *in vitro* study. This would suggest that pol η also has a role in generating the primary mutation observed in cellular studies, G→T transversions. However, in the previous *in vitro* study, pol η was not capable of extending from the mispaired dG-C8-IQ:A terminus. TLS by pol η was examined from the primer terminus dG-C8-IQ:N, where N is C, A, or T to further elucidate the polymerases role in TLS of dG-C8-IQ (**1.26**) (Figure 2-08). At G_3, pol η was ~2-fold more efficient in extending from the dG-C8-IQ:C pair than from the mispairs with A or T (46% versus 28 and 21%), thus the correct

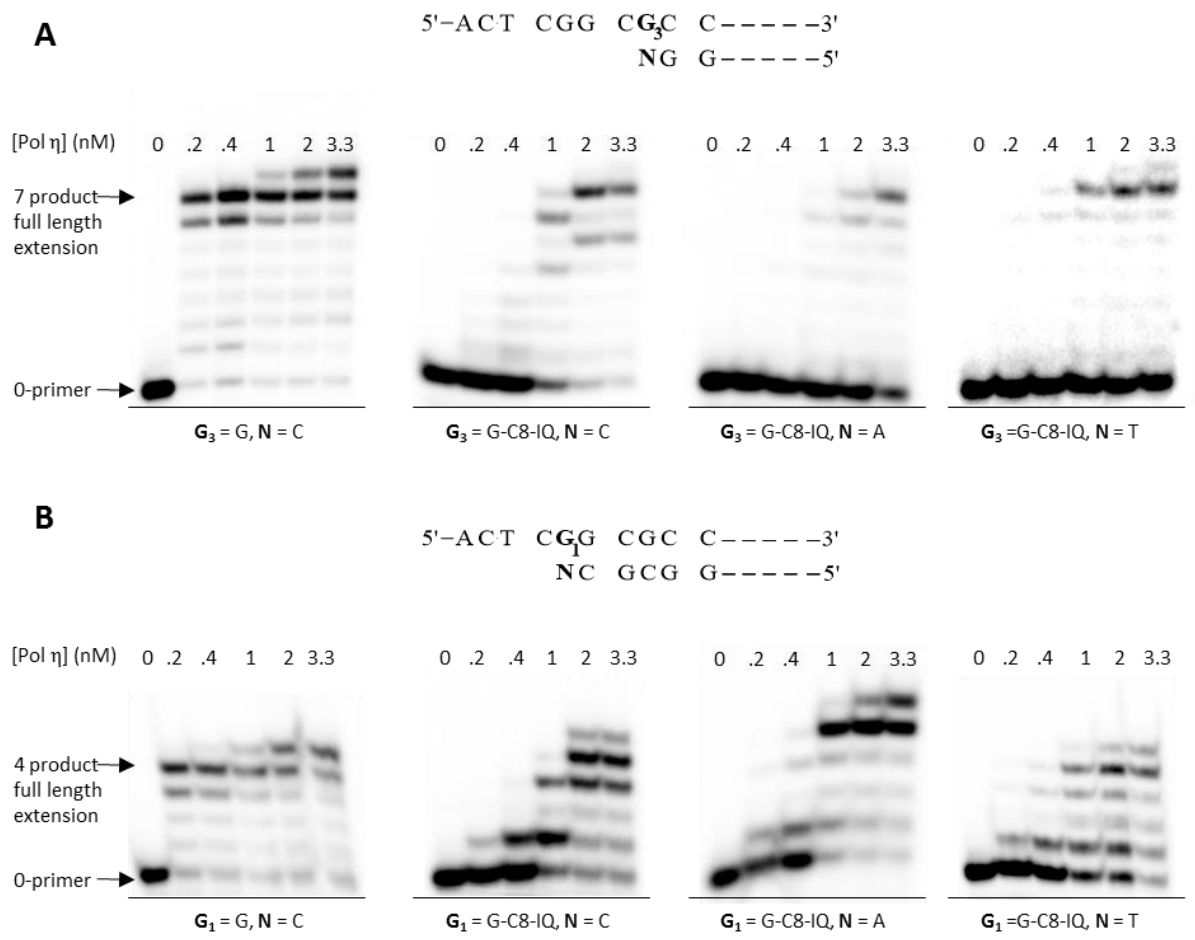

Figure 2-08: *In vitro* extension assay of the dG-C8-IQ adduct by pol ι. (A) Extension of the dG_3-C8-IQ adduct when paired with C, A, or T after 5h at 37^0C. (B) Extension of the dG_1-C8-IQ adduct when paired with C, A, or T after 5h at 37^0C. The DNA was 10 nM.

extension from the dG_3-C8-IQ:N, where N is C, A, or T, primer terminus. Pol η was observed to extend from dG_1-C8-IQ:N primer terminus, where N is C, A or T, to more than full length (Figure 2-08B). Interestingly, the extension from the dG_1-C8-IQ:C was not favored over extension from the mispair of A or T with the adduct at G_1, which contradicts the cellular results. Extension from dG-C8-IQ:A was observed as 71% at full length versus extension from T or C at 43% or 38% respectively. These results suggests pol η also contributes to the G→T transversions and the G→A transitions observed in cellular studies.[8] Pol η extended from dG_1-C8-IQ mispaired with either A or T. The results for the i*n vitro* extension by pol η of dG_3-C8-IQ:N, where N is C, A, or T, were in agreement with previous *in vitro* insertion and extension results

and cellular results, which indicated pol η primarily yielded error-free TLS of dG_3-C8-IQ:C for the adduct at G_3. The disagreement between *in vitro* and cellular studies when dG-C8-IQ (**1.26**) is at G_1 of the *Nar*I sequence may be due to the differences in environment and the accessory proteins present in cells to assist in TLS. Overall, the previous and current results indicate pol η may contributed to base substitution mutations through the insertion of dATP opposite of the dG-C8-IQ (**1.26**) adduct and through extension from the primer terminus dG-C8-IQ:N, where N is A or T.

Summary: the Role of TLS Polymerases Contributing to dG-C8-IQ Mutagenesis

Bulky DNA adducts such as dG-C8-IQ (**1.26**) are known to block DNA replication. The C8 and N^2-IQ adducts of dG are a strong block to *in vitro* TLS by pol δ.[4] In addition, the carbocyclic anaolog of dG-C8-IQ (**1.26**) inhibited replication by *E. coli* DNA polymerase I, exo-free Klenow fragment, exo-free DNA polymerase II, and Dpo4.[16] Previously, human pol η was the only pol identified to efficiently extend primers beyond the dG-C8-IQ (**1.26**) adduct site *in vitro*, where pol κ and ι were shown to insert opposite the lesion followed by little to no extension.[16]

In recent studies, TLS pols ζ, Rev1, κ, ι, and η are implicated in the TLS of the C8-dG lesion of the probable carcinogen 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) through siRNA knockdown of the TLS pols in HEK293T cells.[17,8] However, TLS was not wholly dependent upon anyone polymerase as was the case for the C8-dG adduct of 3-nitrobenzanthrone (dG-C8-ABA).[18] The TLS of the dG-C8-IQ (**1.26**) lesion was examined at each guanine in the *Nar*I recognition sequence, a hotspot for frameshift mutations, where the dG-C8-IQ (**1.26**) lesion is known to be intercalated at G_3 and located in the minor groove at G_2 and G_1.[12] The efficiency of

TLS pols in processing the dG-C8-IQ (**1.26**) lesion and the mutagenicity at the respective adducted guanine were the only clear discrepancies between the locations of the lesion in the *Nar*I site.[8] The percentage of TLS and the level of mutagenicity were observed as $G_3 > G_2 > G_1$.[8] The variance in the efficiency of TLS and mutagenicity observed in HEK293T cells may be due to the difference in structure between the adduct located at G_3 vs G_2 and G_1. It is possible that large active sites of TLS pols accommodate various conformations of a DNA adduct in their active site involving both Watson−Crick and non-Watson−Crick hydrogen bonding to bypass bulky lesions.

In cellular studies dG-C8-IQ (**1.26**) induced primarily G→T transversions and a minority of G→A transitions in HEK293T cells; importantly, no frameshift mutations were observed.[8] siRNA knockdown studies suggest that pol η is essential for the error-free bypass of the dG-C8-IQ (**1.26**) lesion in HEK293T cells, while pol κ and ζ were observed to be the primary polymerases involved in error-prone TLS. Thus, pols ζ and κ are implicated in the generation of the observed base substitution mutations in the cellular study in HEK293T cells.[8] Pol η and ι were previously related to the observed mutations through the observation that pol η will insert dATP and pol ι will insert dTTP opposite of the dG-C8-IQ (**1.26**) *in vitro*. However, when misinsertion occurred, pol η and ι did not extend from the misinserted base.[4] Therefore, the polymerases responsible for extending the dG-C8-IQ (**1.26**) mispaired primer termini were unknown. The role of TLS polymerases ζ, Rev1, κ, ι, and η in contributing to the G→T transversions and G→A transitions were further examined through *in vitro* extension from the primer terminus dG-C8-IQ:N, where N is C, A, or T at both the G_1 and the iterated G_3 position of the *Nar*I site. Pol ζ and Rev1 were also evaluated for the ability to insert nucleotides opposite of the dG-C8-IQ (**1.26**) lesion, and were found incapable of insertion, *in vitro* (Figure 2-04). The

lack of insertion by pol ζ is consistent with reports that pol ζ acts primarily as an extender.[19, 20] In the *in vitro* assay, pol ζ was observed to extend ~4 fold more efficiently from A vs C at G_3, which is consistent with role in error-prone TLS implied by the study HEK293T cells (figure 2-05).[8] Interestingly, pol κ was very inefficient at extending from the dG-C8-IQ:N, where N is C, A, or T, primer terminus at either G_3 or G_1 (figure 2-06A and B). Minimal extension by pol κ from A and T was observed at G_3 as well as from A at G_1. Insertion of the nucleotide after the adduct (+1 primer position) was suspected to be rate limiting for pol κ. Pol κ was then shown to extend relatively efficiently from the +1 primer position after the dG-C8-IQ:A mispair (Figure 2-06C and D). The result suggests the cooperation of additional polymerases to participate in error-prone TLS of dG-C8-IQ (**1.26**). This is consistent with the cellular assay, which observed a synergistic decrease in MF upon double knockdown of pols κ and ζ.[8] In the *in vitro* assay, pol η was observed to participate more efficiently in error-free TLS at G_3 but not at G_1. The results for *in vitro* TLS by pol η at G_1 represent a significant discrepancy in comparison to the cellular study where and increase in MF at G_1 was observed upon knockdown.[8] This discrepancy may be due to difference in environment in cellular studies compared to *in vitro* and to the presence of accessory proteins participating in TLS in cells. Our results for pol ι, where minimal extension from dG_1-C8-IQ:A was observed, were consistent with pol ι playing a minor role in TLS of dG-C8-IQ (**1.26**) as suggested by the cellular study. In the *in vitro* assays, there was at least some extension from dG-C8-IQ:N, where N is A or T, by TLS pols ζ and η, as well as, extension by pol κ from mispairs from the +1 position, 5' to the lesion. Overall, the *in vitro* assay indicates that each pol investigated has at least some participation in error-prone TLS, which is consistent cellular study.[8]

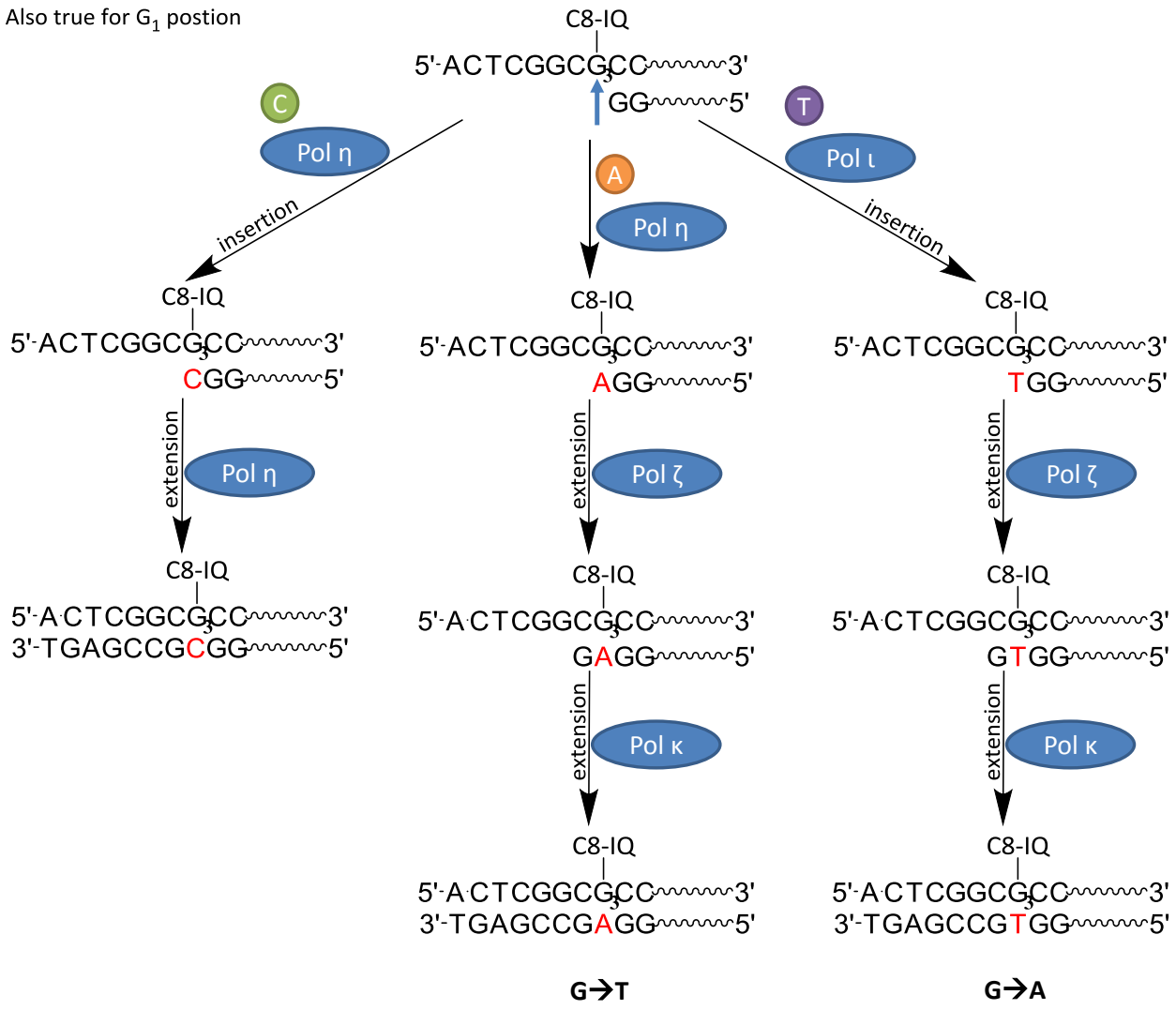

Figure 2-09: Primary results for the TLS of dG-C8-IQ.

The results of the previous and current *in vitro* TLS assays in combination with the cellular assay in HEK293T cells suggest the primary mutation observed G→T transversions arise from the misinsertion of A opposite of dG-C8-IQ (**1.26**) by pol η followed by the cooperative extension from the lesion site by pols ζ and κ (Figure 2-09). The compiled data also indicates the minor mutation, G→A transitions, observed results from misinsertion of T opposite of dG-C8-IQ (**1.26**) by pol ι followed by the cooperative extension by pols ζ and κ (Figure 2-09). In general, *in vitro* assays suggest extension from T is less efficient than extension from A and is likely the reason G→T transversions are predominating.

Experimental Procedures

Materials. yPol ζ, hPol κ, and hpol ι were purchased from Enzymax (Lexington, KY). hRev1 was either a gift from the laboratory of F. P. Geungerich (Vanderbilt University, Nashville, Tn) or purchased from Enzymax (Lexington, KY). The catalytic core of hPol η was a gift from the laboratory of Martin Egli at Vanderbilt University (Nashville, TN). The dNTP solutions (100 mM) were purchased from New England Biolabs (Ipswich, MA) or GE Healthcare (formerly Amersham Biosciences, Piscataway, NJ). [γ-^{32}P]ATP was purchased from Perkin Elmer (Waltham, MA). dG-C8-IQ (**1.26**) modified oligonucleotides were prepared as previously reported.[4] Unmodified oligonucleotides were purchased from Midland Certified Reagents (Midland, TX).

Labeling and Annealing of Oligonucleotides. The primer was 5´ end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (New England Biolabs, Ipswich, MA) according to the manufacturer's instructions and purified on a Biospin column (BioRad, Hercules, CA). Each template and the respective ^{32}P-labeled primer (1:1 molar ratio) were annealed in Tris-HCl buffer (50 mM, pH 7.5) by heating at 90 °C for 5 min and then slowly cooling to room temperature (r. t.) in accordance with previously published procedure.[4]

Single-Nucleotide Incorporation Assays. ^{32}P-labeled primers were annealed to either the unmodified or the dG-C8-IQ (**1.26**) modified template, and extension reactions were then carried out in the presence of single dNTPs. All reactions were initiated by the addition of the dNTP solution (100 μM) to preincubated enzyme/DNA mixtures giving a final reaction volume of 20 μL. The final concentrations of the components for the incorporation assays were in Tris-HCl (50 mM, pH 7.5), DNA duplex (10 nM), ypol ζ (2.5, 5, or 10 nM) or Rev1 (2.5, 5, or 10 nM), dithiothreitol (DTT, 5 mM), bovine serum albumin (BSA, 50 μg/mL-1), NaCl (50 mM),

and $MgCl_2$ (5 mM). The ypol ζ reactions were run at 37 °C for 2 hr. Reactions were quenched with equal volume of EDTA (20 µL, 20 mM) in 95% formamide (v/v) containing xylene cyanol and bromophenol blue dyes. Aliquots (20 µL) were separated by electrophoresis on a denaturing gel containing urea (8.0 M) and 16 % acrylamide (w/v) (from a 19:1 acrylamide/bisacrylamide solution, AccuGel, National Diagnostics, Atlanta, GA) with Tris borate buffer (80 mM, pH 7.8) containing EDTA (1 mM). The gel was exposed to a PhosphorImager screen (Imaging Screen K, Bio-Rad) overnight. The bands were visualized with a PhosphorImaging system (Bio-Rad, Molecular Imager FX) using the manufacturer's Quantity One software, version 4.3.0.

Full-Length Extension Assay with All Four dNTPs. The unmodified or dG-C8-IQ (**1.26**) modified template was annealed to the ^{32}P-labeled 0-primers (with a 3'-C, A, or T) or +1-primer and extended in the presence of all four dNTPs (100 µM each) at 37 °C. Reactions time were 5 hr for pols ζ and κ, and 30 min for pol ι and the catalytic core of hpol η. Each reaction was initiated by adding the mixture of the dNTP solution to a preincubated enzyme/DNA mixtures in Tris-HCl (50 mM, pH 7.5) buffer containing DNA duplex (10 nM), ypol ζ (0.2, 0.4, 1.0, 2, and 3.3 nM), hPol κ 0-primer (2.0, 3.3, 5.0, 10, 30 nM), hPol κ +1-primer (0.2, 0.4, 1.0, 2, and 3.3 nM), hpol ι (0.2, 0.4, 1.0, 2, and 3.3 nM), or hpol η (0.2, 0.4, 1.0, 2, and 3.3 nM), DTT (5 mM), BSA (50 µg/mL), NaCl (50 mM), and $MgCl_2$ (5 mM), giving a final reaction volume of 20 µL. Reactions were quenched by the addition of equal volume of EDTA (20 mM) in 95% formamide (v/v) containing xylene cyanol and bromophenol blue dyes. Aliquots (20 µL) were separated by electrophoresis on a denaturing gel containing urea (8.0 M) and 16 % acrylamide (w/v) (from a 19:1 acrylamide/bisacrylamide solution, AccuGel, National Diagnostics, Atlanta, GA) with Tris borate buffer (80 mM, pH 7.8), containing EDTA (1 mM). Gels were exposed to a PhosphorImager screen (Imaging Screen K, Bio-Rad) overnight. The bands were visualized with

a PhosphorImaging system (Bio-Rad, Molecular Imager FX) using the manufacturer's Quantity One software, version 4.3.0.

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CHAPTER III

REPLICATION OF THE N^2-DEOXYGUANOSINE IQ ADDUCT

Introduction

TLS across Bulky N^2-alkyl Guanine DNA Adducts by Pol δ, ι, η, and κ

A series of studies have examined the TLS of several bulky N^2-alkyl guanine DNA adducts.[1, 2, 3] The results may lend insight into the TLS of dG-N^2-IQ (**1.27**) adduct which was shown to undergo slower repair than dG-C8-IQ (**1.26**) and contributes to carcinogenesis induced by IQ in laboratory animals.[4, 5, 6] A set of seven N^2-alkyl or aryl adducts ranging in size from the smallest N^2-methyl-dG (N^2-Me-dG) to the largest N^2-methyl(6-benzo[*a*]pyrenyl)-dG (N^2-BPdG) were evaluated for the effect of their bulk on TLS by DNA pols δ, ι, η, and κ.[1, 2, 3] Pol δ with PCNA was the most affected by adduct size. Pol δ/PCNA was hindered from bypassing lesions larger than N^2-Me-dG and N^2-ethyl-dG (N^2-Et-dG), which were bypassed in an error free manner. Pol ι, η, and κ were able to bypass lesions smaller than N^2-methyl(2-naphthyl)-dG (N^2-Naph-dG), where pol ι bypass was error prone pols η and κ bypass were primarily error-free. Error-prone bypass by pol ι is probably related to the polymerase bypass utilizing Hoogsteen base pairing.[2, 3] Pol η and κ were the only polymerases active *in vitro* for the bypass of adduct larger than N^2-methyl(9-anthracenyl)-dG (N^2-Anth-dG). However, with the larger adduct size pol η bypass was primarily error-prone, while pol κ bypass was relatively error-free.[1, 2, 3] Overall, the authors of the study concluded that pol κ may be considered the most efficient and accurate TLS pol for bypass of bulky N^2-guanine minor groove DNA adducts. In making comparisons to the above study, there are key differences that should be noted.[1, 2, 3] The alkyl and aryl alkyl N^2-

guanine adducts considered in the above study are more hydrophobic than our aryl amine of interest dG- N^2-IQ. In addition, the structure of dG-N^2-IQ (**1.27**) at G_1 and G_3 of the *Nar*I recognition site, (5′-$G_1G_2CG_3$CC-3′), has been reported and the lesion was intercalated and in the anti-conformation at both positions, not in the minor groove.[7, 8] The anti-conformation of the dG-N^2-IQ (**1.27**) about the gylcosyl bond precludes the possibility of Hoogsteen base pairing unless the polymerase instigates conformational changes.

TLS: Incorporation of Nucleotides Opposite of dG-N^2-IQ

The N^2 deoxyguanosine adduct of IQ is less prevalent than the C8-adduct in reaction with DNA. However, the dG-N^2-IQ (**1.27**) adduct accumulates *in vivo* due to slower repair.[4, 5, 6] The *in vitro* replication of the dG-IQ adducts has been investigated previously. The IQ lesions were site specifically incorporated into 27mer templates at the G_1 and G_3 positions of the *Nar*I recognition site.[6] As discussed in Chapter 1, sequences with GC dinucleotide repeats are susceptible to frameshift mutations. As a result, these sequences are of interest to determine if the DNA adducts of interest are capable of inducing frameshift mutations. For example, the replication of the dG-AAF adduct, especially when located at the iterated G_3 positon (5′-$G_1G_2CG_3$CC-3′), is susceptible to frameshift mutations in *E. coli*.[9, 10, 11] The *in vitro* replication of dG-N^2-AAF in a non-dinucleotide repeat sequence by pol η resulted in a small number of frameshift mutations.[12] However, dG-C8-AAF and dG-N^2-AAF induce only base substitutions in simian (human embryonic) kidney cells.[9, 10, 11, 12] The previous studies revealed the ability of TLS polymerases η, κ, ι and the B-family polymerase δ/PCNA to incorporate nucleotides opposite of and past both the dG-C8-IQ (**1.26**) and dG-N^2-IQ (**1.27**) lesions. Replication by pol δ is completely blocked by the IQ adducts. When present in the *Nar*I restriction site, human pol η extended primers beyond

dG-N^2-IQ (**1.27**) more efficiently than pol κ and much better than pol ι.[6] TLS of the dG-N^2-IQ (**1.27**) lesion at the iterated G_3 position by pol η resulted in –2 deletion products identified by liquid chromatography/mass spectrometry (LC/MS). At the G_1 position, insertion and extension by pol η was observed up to 4 bases utilizing ^{32}P-labeled primers separated by gel electrophoresis to visualize extension. However, the analysis of extension products by LC/MS primarily yielded primer where dCTP was inserted opposite of the dG-N^2-IQ (**1.27**) with no further extension beyond dG-N^2-IQ:C. Pol η was observed to insert dCTP and dATP opposite of the dG-N^2-IQ (**1.27**) lesion, and the misinsertion frequency of dATP by pol η was 0.71 at G_1 and 0.042 at G_3. However, no extension was observed, when pol η inserted dATP opposite of the lesion.[6] Human pol κ was capable of inserting dCTP and dGTP opposite of the dG-N^2-IQ (**1.27**) adduct, then extension of a few base pairs was observed but not to full-length.[6] The misinsertion frequency of dGTP by pol κ was .0023 at G_1 and .047 at G_3. The same study revealed human pol ι would insert dCTP or dTTP opposite of dG-N^2-IQ (**1.27**) lesion.[6] Pol ι was incapable of further extension following insertion, and the reported misinsertion frequency of dTTP was 0.56 at G_1 and 0.33 at G_3.[6] Of the polymerases examined, the pol η was the most efficient in the insertion of dCTP opposite of dG-N^2-IQ (**1.27**) *in vitro*. The misincorporation results of this *in vitro* study suggested that TLS of dG-N^2-IQ (**1.27**) could result in G→A transitions, or the transversions G→T, G→C.[6] Further studies in HEK93T cells would reveal that mutations observed in HEK293T cells from replication of dG-N^2-IQ (**1.27**) were G→A and G→T point mutations.[13] Thus, the *in vitro* insertion of dGTP by pol κ was irrelevant to the mutagenesis induced by the lesion.

Induced Mutagenesis siRNA knockdown of TLS Polymerases

The mutagenesis of the dG-N^2-IQ (**1.27**) adduct in HEK293T (human embryonic) kidney cells was investigated utilizing siRNA knockdown of the TLS polymerases. The study was performed in the laboratory of Ashis K. Basu. This study specifically investigated the role of various TLS polymerases η, κ, ι, ζ, and hRev1 in bypassing dG-N^2-IQ (**1.27**) located at the three guanine positions of the *Nar*I restriction site in human cells.[13] In this particular investigation, the siRNA knockdown of the targeted TLS polymerase(s) resulted in at least 70% silencing. The negative control, where TLS was not reduced, revealed that the percentage of TLS of dG-N^2-IQ (**1.27**) was greater G_1 versus G_2 or G_3, $G_1 > G_2 > G_3$ (Figure 3-01). Each of the TLS pols the reduction in the %TLS relative to wild-type (WT) for each knockdown performed (Figure 3-01). The simultaneous knockdown of pol η/pol ζ/hRev1 showed the most pronounced effect on

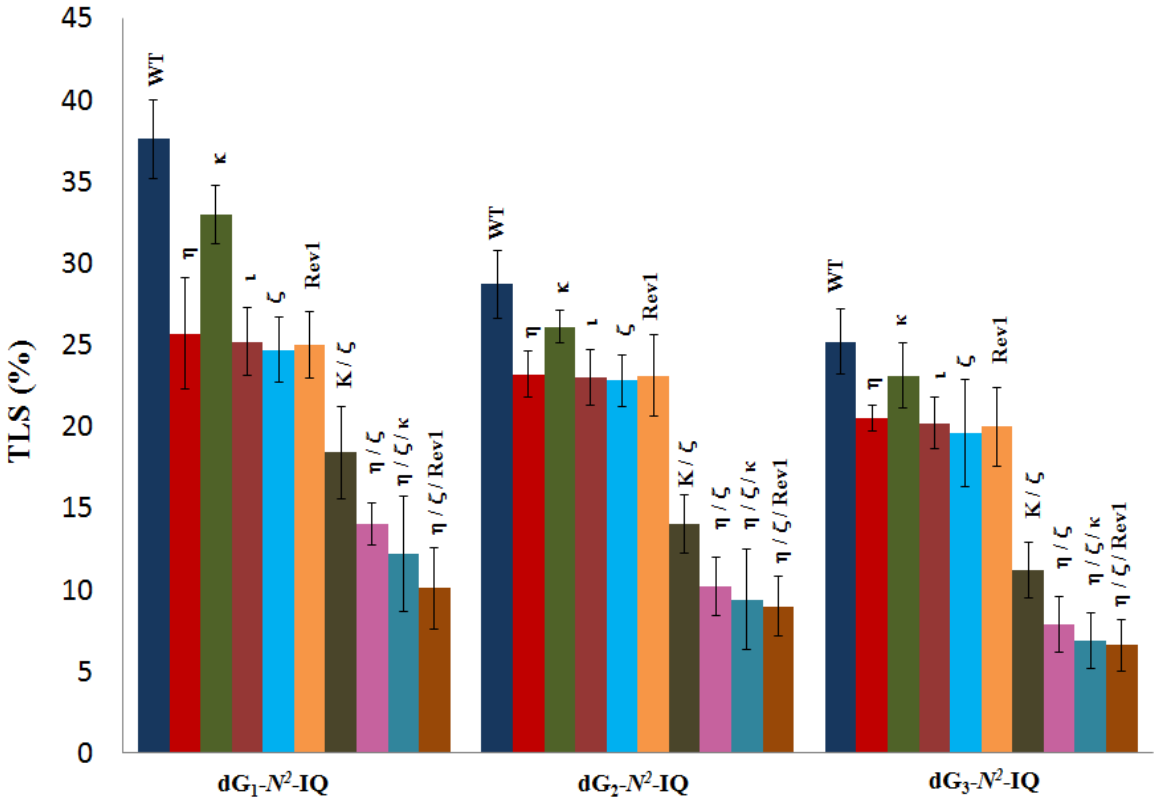

Figure 3-01. The effect of the siRNA knockdowns of TLS polymerases on the percentage of replicative bypass of the dG-N^2-IQ at G_1, G_2 and G_3 of the oligonucleotide constructs in HEK293T cells. The cells were transfected with NC siRNA (WT) or siRNA for single, double or triple pol(s) knockdowns (according to each bar label).

reduction in TLS indicating significant involvement of these polymerases in processing the dG-N^2-IQ (**1.27**) lesion. Considering the individual knockdown of the polymerases, reduction in TLS evaluated were involved in replication of dG-N^2-IQ (**1.27**) adduct to some extent as indicated by was nearly equivalent upon knockdown of η, ζ, ι, or hRev1, which suggests that multiple polymerases can process the lesion or that two or more polymerases act cooperatively to bypass the lesion. Of the pols analyzed, the knockdown of pol κ stands out as the observed reduction in TLS was much less than for the other polymerases. The primary mutations observed were G→T transversions resulting from the misinsertion of A; however, some G→A transitions were also seen. In contradiction to the *in vitro* analysis, no frameshift mutations were observed. The analysis also showed that dG-N^2-IQ (**1.27**) is mutagenic in HEK293T cells at all three sites (Figure 3-02). The order of mutational frequency (MF) for each adducted G was $G_1 > G_2 > G_3$. Thus, the greatest number of mutations are observed at G_1, where the highest percentage of TLS occurs. Knockdown of pol κ resulted in an increase in MF, which was most pronounced at G_1 (Figure 3-02). Therefore, pol κ is essential to error free bypass of the dG-N^2-IQ (**1.27**) lesion as there are fewer mutations during TLS by active pol κ. In contrast, MF at each site was decreased when pol κ, ι, ζ or Rev1 were knocked down (Figure 3-02). Thus, these polymerases are collectively responsible for the error-prone TLS of dG-N^2-IQ (**1.27**). The most pronounced decrease in MF at each site was observed upon knockdown of pol η especially at G_1 and G_3. In addition, the simultaneous knockdown of two polymerases showed that the lack of each two or three pol combination resulted in further decrease in MF indicating a more error prone process. For example, a decrease in MF was observed when pols κ and ζ were simultaneously knocked down (Figures 3-02), where the individual polymerases exhibited opposite effects. In addition, the simultaneous knockdown of pol ζ and Rev1 together, and that of pol κ, ζ, and Rev1 together,

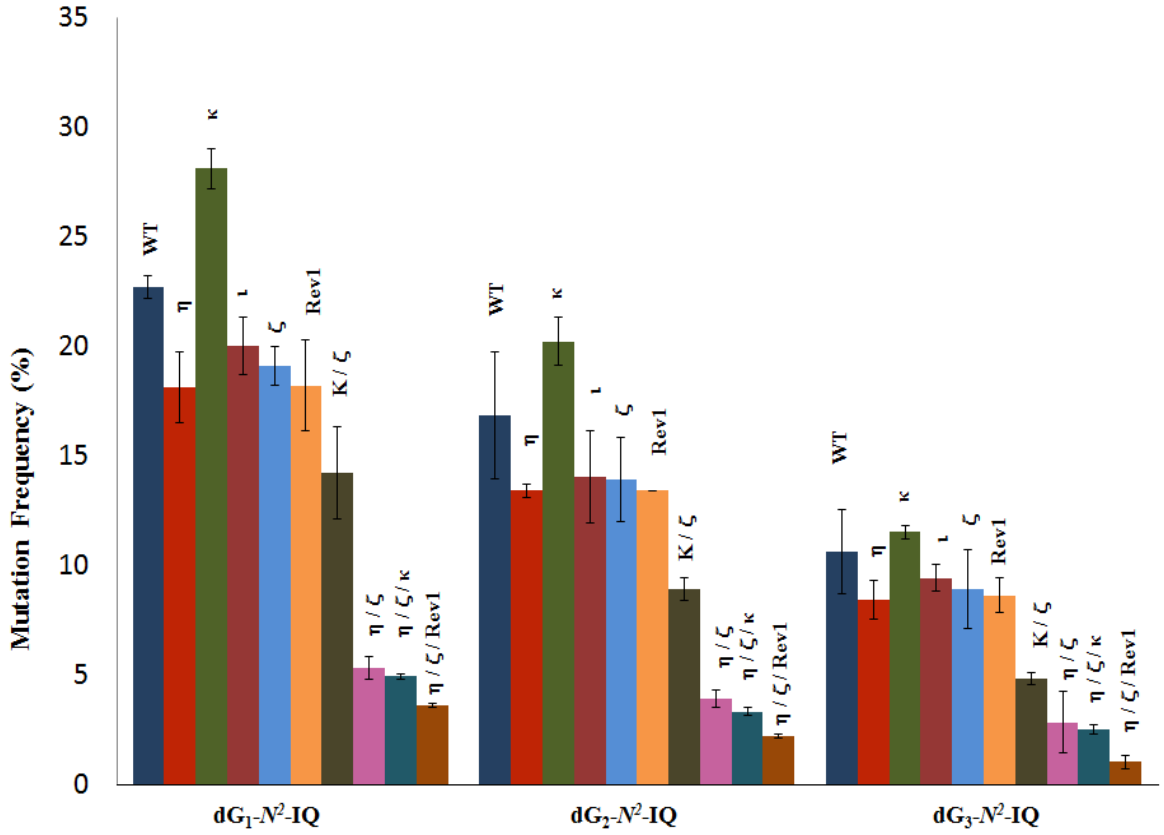

Figure 3-02. The depicted results represent the mutational frequency resulting from TLS of dG-N^2-IQ at G_1, G_2 and G_3 of the oligonucleotide constructs in HEK293T cells where the cells were transfected with NC siRNA (WT) or siRNA for single, double or triple pol(s) knockdowns (according to each bar label).

decreased MF at each adducted guanine in the sequence much more than any one pol individually (Figure 3-02). Simultaneous knockdown of pols η/ζ/hRev1 exhibited a remarkable synergy on the reduction of MF, which indicates that the polymerases may cooperate in processing dG-N^2-IQ (**1.27**) lesion (Figure 3-02). The results of this study suggest the most critical role in the error-prone TLS of the dG-N^2-IQ (**1.27**) adduct is played by pols η, ζ, and hRev1, whereas pol ι likely has a relatively minor role in error-prone TLS of the N^2 adduct. The study also indicates that pol κ plays a critical role in error-free TLS of the dG-N^2-IQ (**1.27**) lesion.

Overall, Pol κ, η, hRev1, and ζ are the primary TLS polymerases implicated in the bypass of the dG-N^2-IQ (**1.27**) adduct.[13] The siRNA knockdown study also revealed that the primary

mutations induced by dG-N^2-IQ (**1.27**) in HEK293T cells were G→T transversions, and the minor mutation were G→A transitions. No frameshift mutations and no G→C transversions were induced by dG-N^2-IQ (**1.27**) in HEK293T cells.[13] Furthermore, a previous *in vitro* study showed that human pol κ and η extended primers beyond dG-N^2-IQ (**1.27**) adduct at either G1 or G3.[6] This study also established that pol η is capable of the misinsertion of dATP but not extension from dG-N^2-IQ:A base pair, and that pol ι is capable of the misinsertion of dTTP, but not extension from the dG-N^2-IQ:T base pair.[6] Extension from the reported misinsertion of A by pol η would yield the G→T transversions observed in HEK293T cells.[6, 13] Extension from the misinsertion of T by pol ι would yield the G→A transitions observed in HEK293T cells.[6, 13] However, the *in vitro* study suggests that pol η and ι are incapable of extending from the respective mismatch base they insert opposite of the dG-N^2-IQ (**1.27**) lesion.[6] The current study investigates the *in vitro* TLS polymerases involved in extending from the mismatched base pairs, dG-N^2-IQ:A and dG-N^2-IQ:T, that will yield the G→T transversions and the G→A transitions observed in HEK293T cells.

Results and Discussion

TLS by Pol ζ and Rev1

In the previous bypass study, no *in vitro* TLS experiment using pol ζ or Rev1 were conducted. In the current study, the bypass of dG-N^2-IQ (**1.27**) at G_1 and G_3 in the *Nar*I restriction site by these specialized pols was evaluated. A 5′-^{32}P-labeled 19mer or 22mer was annealed to a complementary modified template containing the dG-N^2-IQ (**1.27**) adduct. Primer extension assays were performed with pol ζ in the presence of all four dNTPs to determine the capability of pol ζ to perform TLS of dG-N^2-IQ (**1.27**). The primer extension was performed with

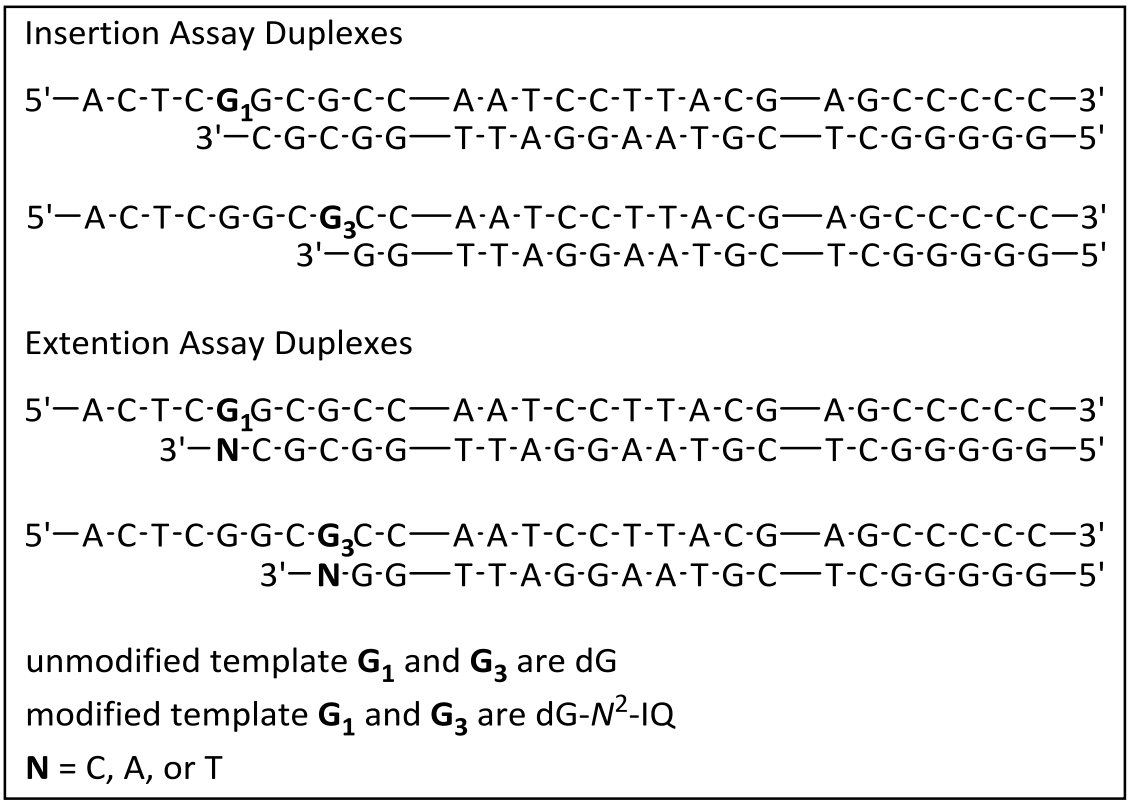

Figure 3-03: Duplexes utilized in TLS assays

Rev1 in the presence of dCTP. Yeast pol ζ was unable to incorporate a nucleotide opposite dG-N^2-IQ (**1.27**) at G_1 or G_3 (Figure 3-04). hRev1 inserted dCTP opposite of dG-N^2-IQ (**1.27**) at both G_1 and G_3 (Figure 3-04B and D). At G_3, hRev1 inserted dCTP twice (Figure 3-04B). This may indicate the slippage of the template with 5′ neighbor C and G_3-N^2-IQ (CG_3-N^2-IQ) bulged outside of the duplex (Figure 3-05). In this case, with CG_3-N^2-IQ bulged outside of the duplex, these base would not be replicated, which would yield a −2 deletion. The lack of nucleotide insertion by ypol ζ is consistent with reports that the primary role of pol ζ in TLS is extension from lesion mispairs generated by other TLS polymerases (Figure 3-04A and C).[14] In addition, insertion of dCTP opposite of dG- N^2-IQ is consistent with reports that Rev1 proficiently incorporates C opposite of various N^2-adducted dGs. Otherwise, Rev1 acts frequently as an accessory protein.[15, 16, 17]

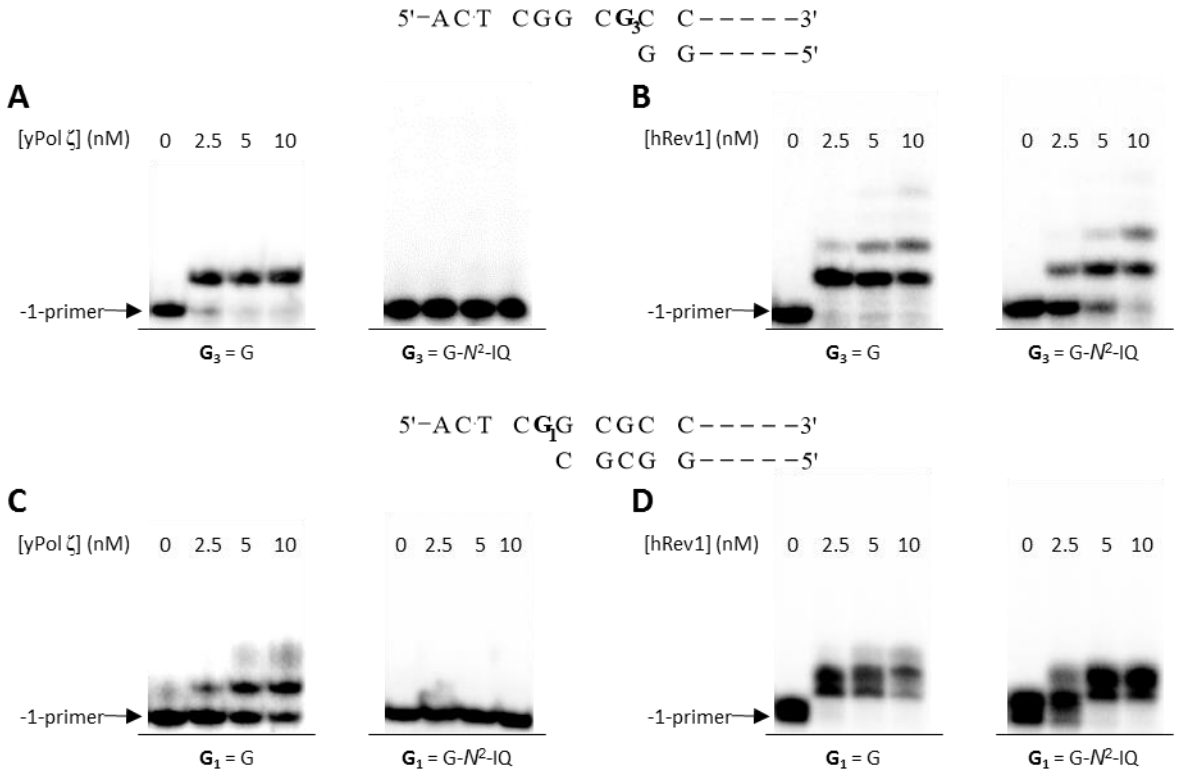

Figure 3-04: In vitro insertion and extension assay of the dG-N^2-IQ adduct by ypol ζ and human Rev1 insertion assay. (A) Insertion by ypol ζ of dCTP opposite a control unmodified dG (left) and reaction of the dG_3-N^2-IQ modified oligonucleotide in the presence of all four dNTPs. (B) Insertion of dCTP (100 μM) opposite a control C (left) or dG_3- N^2-IQ (right) by hRev1 at 37 °C. (C) Insertion by ypol ζ of dCTP opposite a control unmodified dG (left) and reaction of the dG_1-N^2-IQ modified oligonucleotide in the presence of all four dNTPs. (D) Insertion of dCTP (100 μM) opposite a control C (left) or dG_1- N^2-IQ (right) by hRev1 at 37 °C. The DNA concentration was 10 nM.

As pol ζ typically acts as an extender, the role of ypol ζ to extend from a dG-N^2-IQ:N template-primer junction was evaluated (Figure 3-06). Indeed, ypol ζ was observed to extend from dG-N^2-IQ:N base pairs at G_1 and G_3, where N is C, A or T, by at least one nucleotide. The three primers represent the major replication outcomes observed in the cell mutagenesis studies. Interestingly, pol ζ extended from the mispair of dG-N^2-IQ (**1.27**) with T or A more readily than from C at both G_1 and G_3. The efficiency of extension by one base is more efficient at G_1 than G_3, where one base extension was T, A (82%) > C (60%) at G_1 and T (57%) > A (38%) > C (20%) at G_3. The results are consistent with the study in HEK293T cells in that the percentage of TLS by pol ζ was greater at G_1 than at G_3 and because the TLS observed by pol ζ was more error-prone

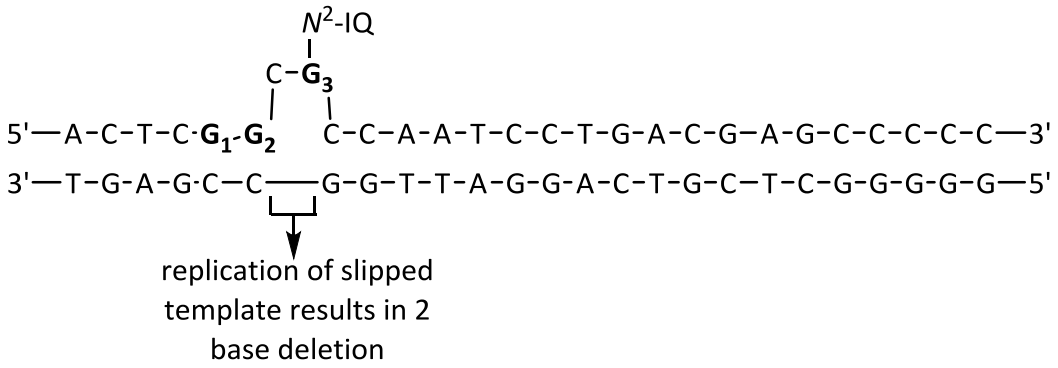

Figure 3-05: Slipped template alignment yielding –2 deletion products is shown. During replication with dG-N^2-IQ and 5′base bulged out of the duplex, after the replicating polymerase inserts opposite of the base 3′ to the lesion, the polymerase next inserts opposite of $\mathbf{G}_2$.

as in the cellular study. The *in vitro* results suggest extension beyond the one nucleotide by ypol ζ at the dG-N^2-IQ:N terminus, where N is A, T, or C, would require the cooperation of another TLS polymerase to extend several base pairs beyond the lesion where normal replication could

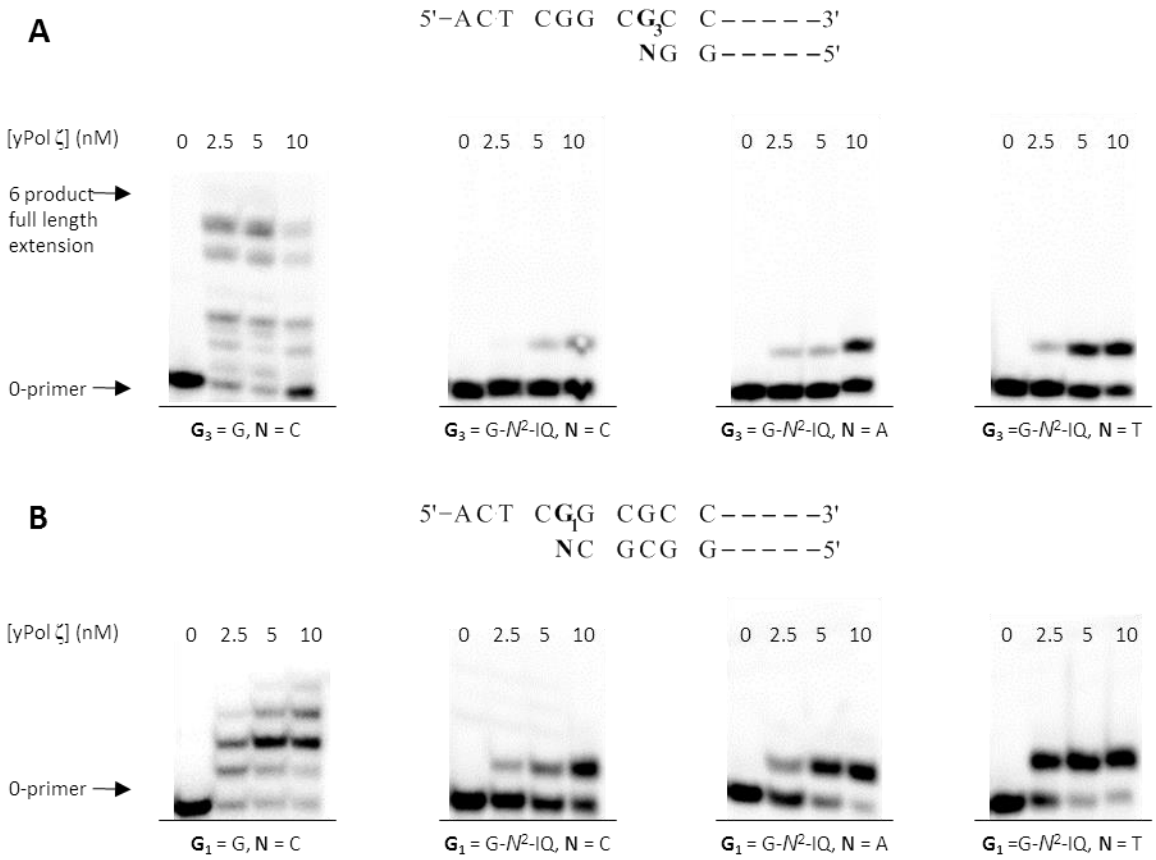

Figure 3-06: *In vitro* extension assay of the dG-N^2-IQ adduct by ypol ζ. (A) Extension of the dG_3- N^2-IQ adduct when paired with C, A, or T after 2 h at 37 °C. (B) Extension of the dG_1- N^2-IQ adduct when paired with C, A, or T after 2 h at 37 °C. The DNA was 10 nM.

resume. The synergistic effect of the reduction in MF by the triple knockdown of pol ζ/η/ hRev1, also suggests cooperation of the polymerases in TLS of the N^2-IQ lesion. The inability of pol ζ to insert nucleotides opposite of dG-N^2-IQ (**1.27**) and the ability to extend from dG-N^2-IQ:N, where N is C, A, or T, is consistent with the reported role of pol ζ as an extender from error free lesion and mispaired lesion termini.[15, 17, 18, 19, 20]

TLS by Pol ι

Cellular studies indicate pol ι plays a minor role in error-prone TLS of dG-N^2-IQ (**1.27**) with MF slightly decreasing upon knockdown.[13] In the previous *in vitro* study, human pol ι was less efficient than pol η and κ. Pol ι was capable of inserting C or T opposite of the lesion, then no further extension was observed.[6] The previous *in vitro* study indicate that the misinsertion of T is one way pol ι contributes to error-prone TLS. The ability of pol ι to extend *in vitro* from dG-N^2-IQ (**1.27**) mismatched primer termini at G_1 and G_3 of the *Nar*I sequence was evaluated (Figure 3-07). Human pol ι extend from the dG-N^2 -IQ:A primer termini by one base pair *in vitro* (Figure 3-07). The extension from G_1 was ~9 times more efficient than from G_3, where the percentage of primer extended by one base was ~90% and 10%, respectively. Pol ι failed to extend from dG-N^2-IQ:N, where N is C or T, as in the previous *in vitro* study. These results agree with the conclusion of the cellular study that pol ι has a minor role in the TLS of the dG-N^2-IQ (**1.27**) adduct, also the percentage of TLS and the MF are greater at G_1 than G_3. TLS of the dG-N^2-IQ (**1.27**) by pol ι was error-prone as in the case of the N^2-alkyl-guanine study discussed above. The differential processing of the dG-N^2-IQ (**1.27**) adduct at G_1 versus G_3 of the *Nar*I site may be related to minor differences in structure where within the duplex, the base pair to G_1, C in the complementary strand, is rotated further out of the duplex then at G_3. In addition, a slight

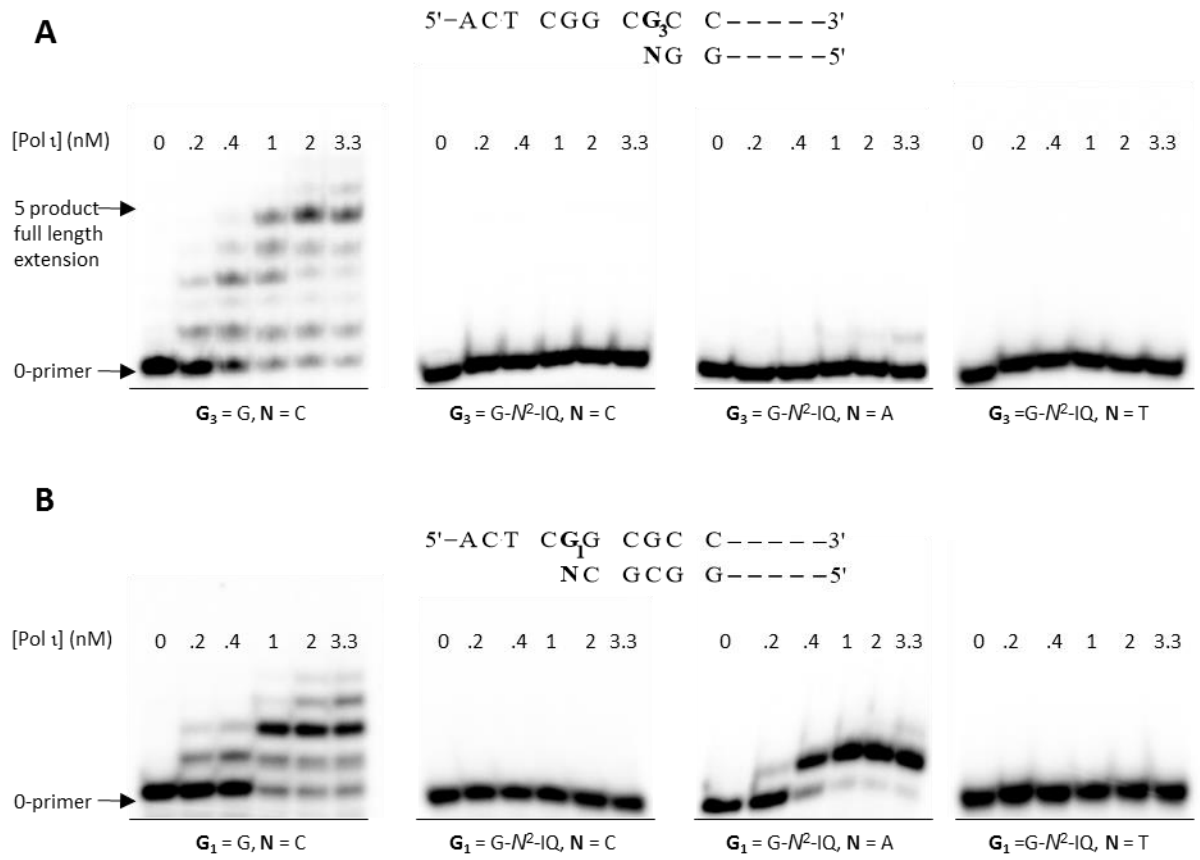

Figure 3-07: *In vitro* extension assay of the dG-N^2-IQ adduct by pol ι. (A) Extension of the dG_3- N^2-IQ adduct when paired with C, A, or T after 30 min at 37 °C. (B) Extension of the dG_1- N^2-IQ adduct when paired with C, A, or T after 30 min at 37 °C. The DNA was 10 nM.

unwinding of theduplex was observed at G_1 but not at G_3.[8] The structural findings suggest the duplex is more distorted at G_1 than G_3, which may affect polymerase recognition and binding.[1] Considering the anti-conformation of the dG-N^2-IQ (**1.27**) lesion, any replication by pol ι isinteresting. Pol ι known for replicating through the syn conformation of adducts, as pol ι utilizes Hoogsteen base pairing during replication.[2, 3, 17, 21]

TLS by Pol η

Previous *in vitro* studies suggests pol η participates in error-prone TLS of dG-N^2-IQ (**1.27**) lesions through the misinsertion of A opposite of dG-N^2-IQ (**1.27**) and frameshift mutations observed as −2 deletion products when N^2-IQ lesion is at G_3.[6] However, frameshift

mutations were not observed in HEK293T cells upon siRNA knockdown of pol η. The discrepancy maybe related to accessory proteins present in cells that may assist in TLS, for example, through preventing the adduct from bulging out of the duplex. The misinsertion of A opposite of the dG-N^2-IQ (**1.27**) lesion and extension from the base pair would account for the G→T transversions observed in HEK293T cells. However, in the previous *in vitro* products representing extension from dG-N^2-IQ:A were not observed upon LC/MS sequencing of the extension products. Furthermore, extension from dG_1-N^2-IQ:C were not observed in the LC/MS sequencing analysis of extension products, yet extension was observed upon utilizing ^{32}P-labeling and gel electrophoresis techniques. The conclusion of the previous *in vitro* study was that pol η could misinsert A opposite of the lesion, but further extension was blocked.[6] Pol η is strongly implicated in the error-prone TLS of the dG-N^2-IQ (**1.27**) adduct by the reduction in MF observed upon siRNA knockdown. In the current, *in vitro* extension from dG N^2-IQ:N, where N is C, A, or T, by the catalytic core of pol η is evaluated when the adduct is at G_1 and G_3 of the *Nar*I recognition sequence (Figure 3-08). In contrast to the previous *in vitro* assay, extension to full-length from dG-N^2-IQ:A was observed at both G_1 and G_3. Pol η also extended from dG-N^2-IQ:T mispair to full-length. Pol η extended more efficiently from dG-N^2-IQ:A than from the mispair with T or correct base pairing with C. At G_3, the extension from dG-N^2-IQ:C was observed to stall after 5 base pairs, which may indicates accumulation of –2 frame shift product reported in the previous study (Figure 3-08A). At G_3, the extension to full-length product by pol η was observed to be ~41% from A, ~21% from C and ~5% from T (Figure 3-08A). At G_1, the extension to full-length product by pol η was observed to be ~71% from A, ~12% from C and ~67% from T (Figure 3-08B). Therefore, TLS by pol η was observed to be primarily error-prone and the percentage of TLS performed was higher at G_1 than G_3, which is in agreement with the

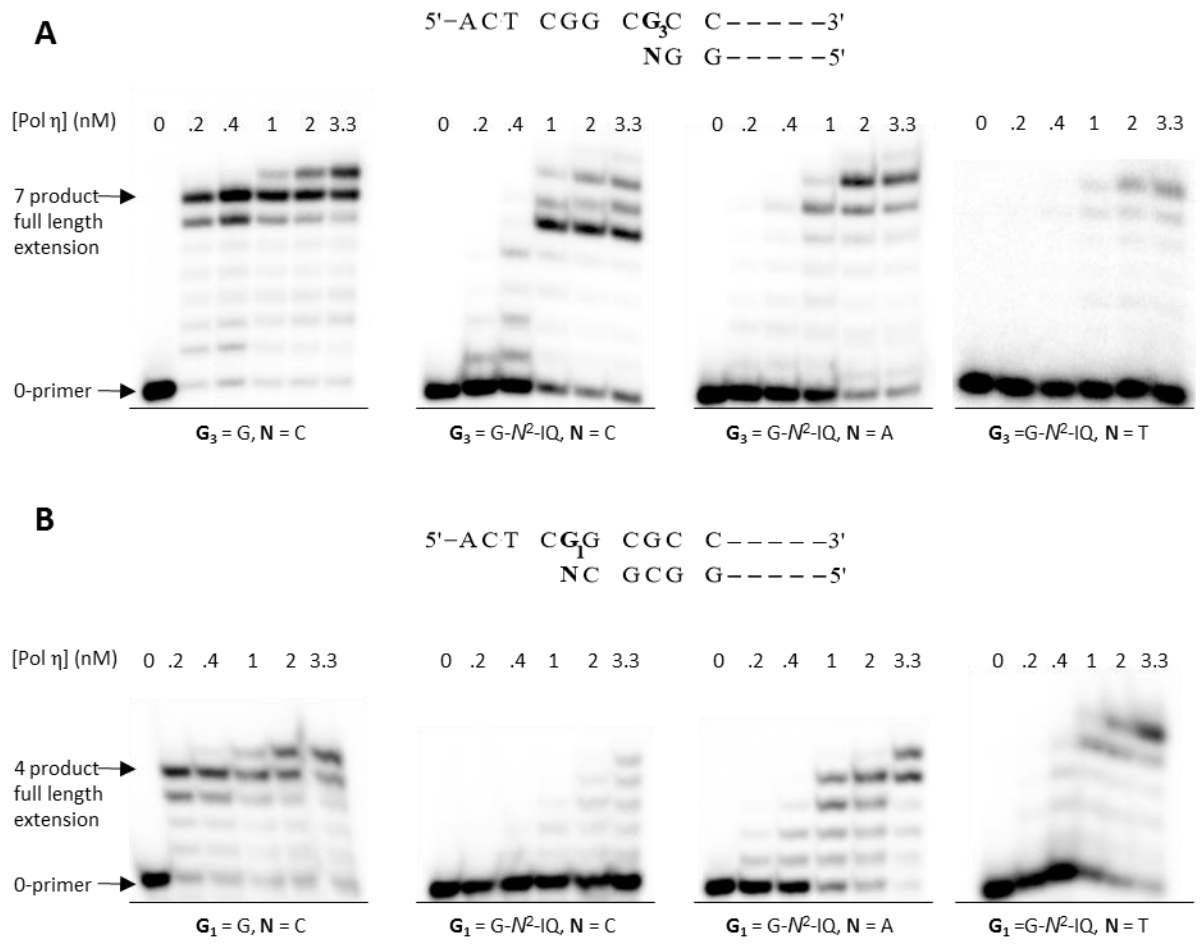

Figure 3-08: *In vitro* extension assay of the dG- N^2-IQ adduct by pol ι. (A) Extension of the dG_3- N^2-IQ adduct when paired with C, A, or T after 30 min at 37 °C. (B) Extension of the dG_1- N^2-IQ adduct when paired with C, A, or T after 30 min at 37 °C. The DNA was 10 nM.

findings of the cellular study in HEK293T cells. Error-prone TLS of dG-N^2-IQ (**1.27**) by pol η also seems to be consistent with the findings of the N^2-alkyl-guanine study. The study reported TLS of N^2 adducts the approximate size of or larger than N^2-Anth-dG by pol η was primarily error-prone. Pol η was found to be capable of extending from dG-N^2-IQ (**1.27**) adducts mispaired with A and T and could therefore contribute to the G→T transversions and G→A transitions observed in HEK293T cells. The –2 deletions observed in the *in vitro* assays were not observed in HEK293T cells. The lack of frameshift mutations in cells is most likely due to cooperation of other TLS polymerases in bypass of the lesion. Possibly, pol η and ζ cooperate in bypass as there

is a synergistic reduction in MF upon double knockdown of pol η/ζ and the triple knockdown of pol η/ζ/Rev1.

TLS by Pol κ

In the previous *in vitro* study, human pol κ was capable of inserting dCTP or dGTP opposite of the dG-N^2-IQ (**1.27**) adduct, and was observed to extend a few base pairs beyond the lesion but not to full-length.[6] Base substitution mutations resulting from the misinsertion of G are not observed in cellular assays, therefore, misinsertion does not account for the role of pol κ in error prone TLS indicated by the results of the cellular assays described above. In HEK293T cells, the MF increased upon siRNA knockdown of pol κ indicating that pol κ is essential to error-free TLS of the dG-N^2-IQ (**1.27**) lesion in *Nar*I sequence. *In vitro* analysis of TLS by human pol κ is reported here to elucidate the role of pol κ in the error-free and error-prone TLS of dG-N^2-IQ (**1.27**) at G_1 and G_3 of the of the *Nar*I restriction site (Figure 3-09). Extension of the dG_3-N^2-IQ:N pair, where N is A or T but not C, by pol κ was observed upto 5 or more base pairs (Figure 3-09A). Pol κ was observed to have difficulty inserting the last two base pairs of the primer sequence at both G_1 and G_3 when paired to unmodified and modified template and is most likely unrelated to lesion bypass. At G_3, the percentage of extension by one or more bases by pol κ, when pol κ is 1 nM, was observed to be ~68% from C, ~31% from A and ~30% from T (Figure 3-09A). At G_1, the percentage of extension by one or more bases by pol κ, when pol κ is 1 nM, was observed to be ~70% from C, ~12% from A and ~60% from T (Figure 3-09B). Therefore, TLS by pol κ was observed to favor error-free bypass, which is in agreement with cellular studies. However, a significant amount of error-prone TLS was observed *in vitro* as pol κ extended from mispairs of dG-N^2-IQ (**1.27**) with both A and T. Therefore, pol κ may contribute

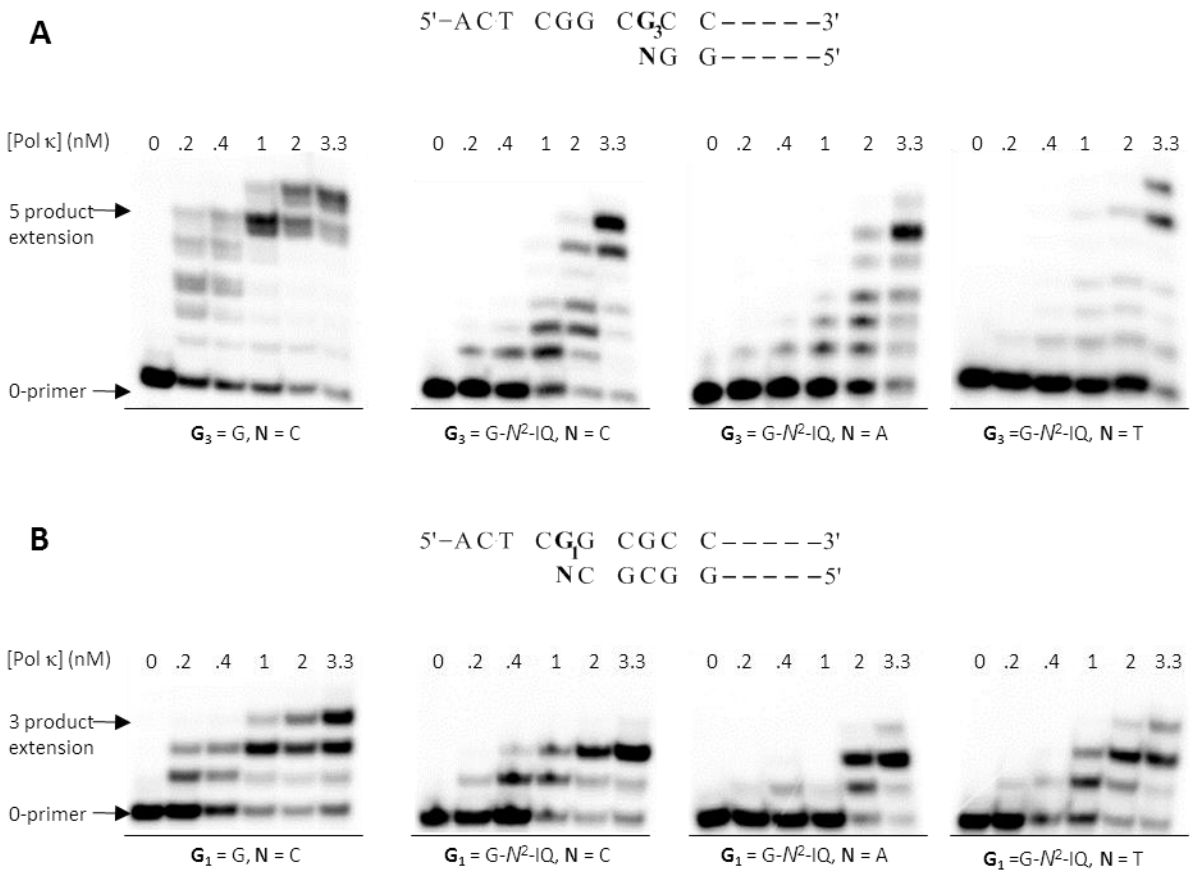

Figure 3-09: (A) Extension past a dG$_3$- N^2-IQ:N pair (N = C, A and T; 10 nM). (B) Extension past a dG$_1$- N^2-IQ:N pair (N = C, A and T; 10 nM). The DNA concentration was 10 nM and was extended by human pol κ after 5 h at 37 °C in the presence of all four dNTPs (100 μM).

to the G→T transversions and G→A transitions observed in HEK293T cells. Pol κ was found to be efficient and accurate in the TLS of the bulky dG-N^2-IQ (**1.27**) intercalated DNA lesion. This finding is similar to the result of pol κ TLS of bulky N^2-guanine minor groove DNA adducts.[3]

Summary: the Role of TLS Polymerases Contributing to dG-N^2-IQ Mutagenesis

Bulky DNA adducts such as dG-N^2-IQ (**1.27**) are known to block DNA replication. The C8 and N^2-IQ adducts of dG are a strong block to *in vitro* TLS by pol δ as are alkyl-guanine adducts larger than N^2-Et-dG.[1, 3, 6] In addition, the aryl amine dG-N^2-AAF inhibited replication by pol α and δ.[12] Previously, human pol η and κ were identified to efficiently extend primers

beyond the dG-N^2-IQ:C base pair *in vitro*, where pol ι was shown to insert opposite the lesion with no further extension.[6]

In recent cellular studies, TLS pols ζ, Rev1, κ, ι, and η are implicated in the TLS of the dG-N^2 lesion of the probable carcinogen 2-amino-3-methylimidazo[4,5-*f*]quinoline through siRNA knockdown of the TLS pols in HEK293T cells. However, TLS was not wholly dependent on any one polymerase as was the case for the C8-dG adduct of 3-nitrobenzanthrone.[22] The TLS of the dG-N^2-IQ (**1.27**) lesion was examined at each guanine in the *Nar*I recognition sequence a hotspot for frameshift mutations. The dG-N^2-IQ (**1.27**) lesion is known to be intercalated at G_3 and G_1 of the *Nar*I sequence.[7, 8] The efficiency of TLS pols in processing the dG-N^2-IQ (**1.27**) lesion and the mutagenicity at the respective adducted guanine were the only clear discrepancies between the locations of the lesion in the *Nar*I site. The percentage of TLS and the level of mutagenicity were observed to follow the order $G_1 > G_2 > G_3$. The variance in the efficiency of TLS and mutagenicity observed in HEK293T cells may be due to the differences in structure of the dG-N^2-IQ (**1.27**) lesion and local sequence surrounding the adduct at each guanine in the *Nar*I site. It is possible that large active sites of TLS pols accommodate various conformations of a DNA adduct in their active site involving both Watson−Crick and non-Watson−Crick hydrogen bonding to bypass bulky lesions such that structure may cause minimal differences in TLS processing.

In cellular studies, dG-N^2-IQ (**1.27**) induced primarily G→T transversions and a minor amount of G→A transitions. Pol κ was observed to be essential to the error-free bypass of the dG-N^2-IQ (**1.27**) lesion in HEK293T cells, while pol η, ζ, and hRev1 were observed to be the primary polymerases responsible for error-prone TLS. Thus, pols ζ and η are implicated in the generation of the observed base substitution mutations in the cellular study, where TLS of dG-

N^2-IQ (**1.27**) is knocked down in HEK293T cells. Pol η and ι were previously related to the observed mutations through their ability to insert dATP and dTTP, respectively, opposite of the dG-N^2-IQ (**1.27**) *in vitro*. However, when misinsertion occurred, pol η and ι did not extend from the misinserted bases. Therefore, the polymerases responsible for extending from the dG-N^2-IQ (**1.27**) mispaired primer termini were unknown. In the present investigation, the role of TLS polymerases ζ, Rev1, κ, ι, and η in contributing to the G→T transversions and G→A transitions is further examined through *in vitro* extension from the primer terminus dG-N^2-IQ:N, where N is C, A, or T at both the G_1 and the iterated G_3 position of the *Nar*I site. Pol ζ and Rev1 were also evaluated for the ability to insert nucleotides opposite of the dG-N^2-IQ (**1.27**) lesion. yPol ζ was found incapable of insertion (Figure 3-04A and C), while hRev1 inserted dCTP opposite of dG-N^2-IQ (**1.27**) at G_1 and G_3 (Figure 3-04B and D). hRev1 insertion of dCTP opposite dG-N^2-IQ at G_3 was followed by a subsequent insertion of dCTP, which may indicate the template slippage allowing –2 deletion (Figure 3-04B and Figure 3-05). Rev1 has previously been reported to replicate through a variety of N^2-guanine lesions, thus Rev1 insertion opposite dG-N^2-IQ (**1.27**) is consistent with previous studies.[15] The lack of insertion by pol ζ is consistent with reports that pol ζ acts primarily as an extender. In the *in vitro* assay, pol ζ extended by one base pair. The trend for extension was A > C > T at G_1 and T > A > C at G_3 (Figure 3-06). Furthermore, pol ζ extended more efficiently at G_1, 82% of 0-primer was extended one base from A, than at G_3 where 57% 0-primer was extended one base from T. The results of the *in vitro* and HEK293T cell study both indicate pol ζ participates in error-prone TLS and that TLS is more efficient and error-prone at G_1 than G_3. Pol ι exhibited one base extension from dG-N^2-IQ:A, which was 9 times more efficient at G_1 than G_3 (Figure 3-07). Extension beyond one base pair by pol ι would require cooperation of other TLS polymerases. Otherwise, the results indicate pol ι plays a minor

role in error-prone TLS of dG-N^2-IQ (**1.27**), as indicated by the lowest reduction of MF upon pol ι knockdown in HEK293T cells (Figure 3-02). In the previous *in vitro* assay, replication of dG_3-N^2-IQ:C by pol η resulted in –2 deletion product. In the *in vitro* assay reported here, pol η was observed to stall after extension from dG_3-N^2-IQ:C by 5 base pairs, which is indicative of a –2 deletion (Figure 3-08A). Pol η was observed to extend the most efficiently from dG-N^2-IQ:A at both G_1 and G_3 (Figure 3-08). In HEK293T cells, the double knockdown of η and ζ greatly reduced MF (Figure 3-02). The results of these two studies imply pol η has a significant role in the error-prone TLS of dG-N^2-IQ (**1.27**). In HEK293T cells, knockdown of pol κ results in an increase in MF, which implies that pol κ is essential to error-free TLS of dG-N^2-IQ (**1.27**) in HEK293T cells. In our *in vitro* assay, pol κ was observed to favor extension from the correct base pairing dG-N^2-IQ:C at both G_1 and G_3 (Figure 3-09). Therefore pol κ was observed to participate primarily in error-free TLS *in vitro*. Although pol κ was observed to favor extension from dG-N^2-IQ:C, pol κ was observed to extend from dG-N^2-IQ (**1.27**) mismatch base pairs. This indicates pol κ may contributes to TLS yielding G→A transversions and G→T transitions observed in HEK293T cells. In the *in vitro* assays, there was at least some extension from dG-N^2-IQ:N, where N is A or T, by TLS pols ζ, η, and κ as well as, extension from dG-N^2-IQ:A by pol ι. Therefore, the *in vitro* assay indicates that each pol investigated has at least some participation in error-prone TLS which is consistent with cellular study.

The results of the previous and current *in vitro* TLS assays in combination with the cellular assay in HEK293T cells suggest the primary mutation observed G→T transversions arise from the misinsertion of A opposite dG-N^2-IQ (**1.27**) by pol η followed by the cooperative extension from the lesion site by pols ζ and η (Figure 3-10). The compiled data also indicates the minor mutation, G→A transitions, observed results from misinsertion of T opposite dG-N^2-IQ

(**1.27**) by pol ι followed by the cooperative extension by pols ζ and η (Figure 3-10). In general, *in vitro* assays suggest extension from insertion of T by pol ι is less efficient than the insertion of A, and the extension from A also appeared more prevalent. This is likely the reason that G→T transversions are predominant over G→A transitions.

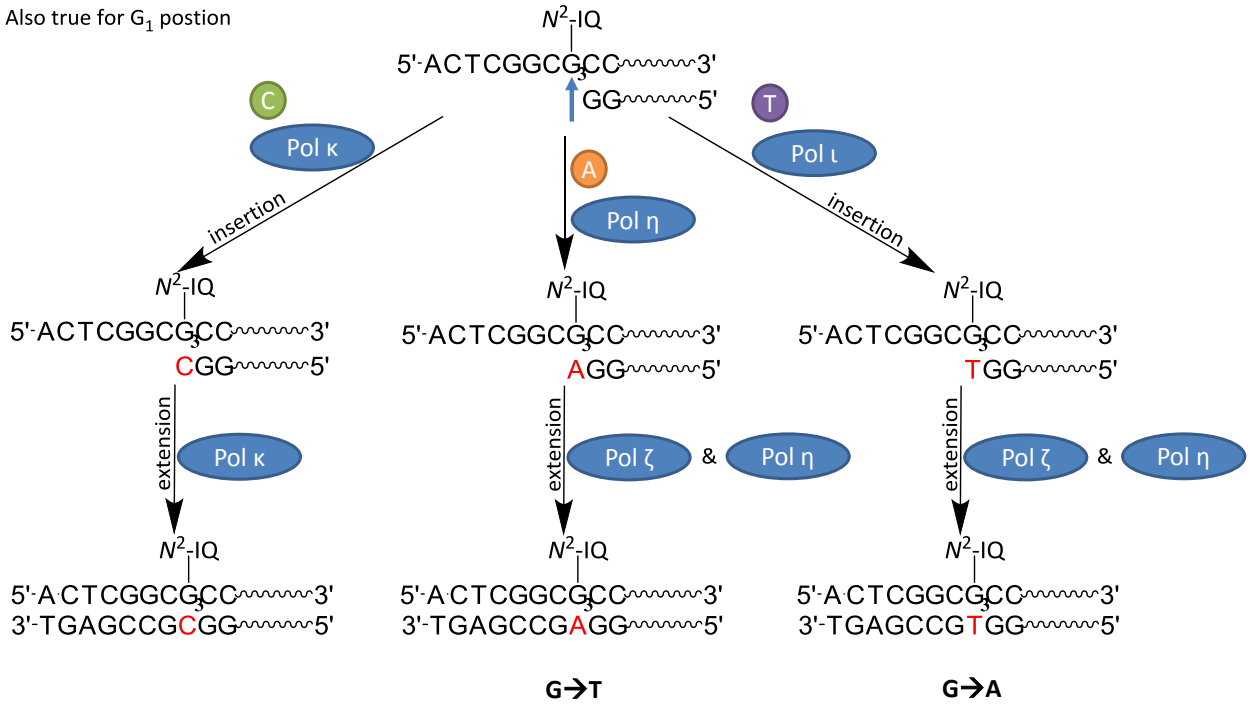

Figure 3-10: Summary primary TLS results for dG-N^2-IQ.

Comparison of the TLS of C8- and N^2-IQ Guanine lesions

The percentage of TLS in HEK293T cells is greater for the C8 adduct than for the N^2 adduct of IQ at G_1, G_2, and G_3 of the *Nar*I site. This indicates that the dG-C8-IQ (**1.26**) is more readily bypassed than dG-N^2-IQ (**1.27**).[13] For the C8 adduct, the structural position of the adduct, minor groove (G_1 and G_2) or intercalated (G_3), was dependent on sequence, but the adduct was in the syn conformation at all positions.[23] However, dG-N^2-IQ (**1.27**) is intercalated at both G_1 and G_3 and in the anti-conformation and only minor local differences in duplex structure are

reported, which indicates that the structural position of dG-N^2-IQ (**1.27**) in the duplex is not greatly affected by sequence.[7, 8] The position of the C8- or N^2-IQ in the *Nar*I sequence had only small impact on the level of TLS performed and the MF observed. This is most likely related to structure of the adduct within the duplex. For the dG-C8-IQ (**1.26**) lesion, the percentage of TLS performed and MF were greatest at G_3 and lowest at G_1, $G_3 > G_2 > G_1$. The trend in percentage of TLS performed and MF was exactly the opposite for the N^2-IQ lesion, $G_1 > G_2 > G_3$. This is most likely related to the structure of the adduct and the local sequence within the duplex. For example, *in vitro* replication of N^2-IQ at the iterated G_3 induced –2 deletions, while C8-IQ did not. The two base deletion *in vitro* assays indicates the template may slip when N^2-IQ is at G_3, meaning that the lesion and 5′ neighbor base are bulged outside of the duplex. A slipped template is likely more difficult for TLS polymerases to process and may account for the level of TLS performed being the lowest in processing dG_3-N^2-IQ. The efficiency of the TLS polymerases required to process either the C8- or the N^2-IQ lesions were affected by the position at which dG was modified within the *Nar*I sequence. Pol η was essential for error free TLS of the C8-IQ lesion, while pol κ was essential for error-free TLS of the N^2-IQ lesion both *in vitro* and in HEK293T cells. The results of the *in vitro* and cellular studies implicated each of the polymerases investigated in the error-prone TLS of IQ adducts to some extent. The error-prone TLS of IQ adducts yielded primarily G→T transversions and to a lesser extent G→A transitions in HEK293T cells. For both dG-C8 and N^2-IQ adducts, replication by pol ι was minor in both *in vitro* and in cellular studies. Pol ι was observed to contribute to error-prone TLS primarily through the insertion of dTTP opposite of the lesion and through one base extension from dG_1-IQ:A and dG_3-N^2-IQ. Interestingly, Rev1 was observed to insert dCTP opposite of dG-N^2-IQ (**1.27**) but not dG-C8-IQ (**1.26**). Rev1 is suggested to favor insertion opposite N^2-guanine

adducts.[15] For the C8-IQ adduct, pol κ and ζ were both essential in error-prone TLS.[13] The results suggest that the polymerases cooperatively bypass the C8 lesion. The *in vitro* results indicate that the insertion of the first primer base 3′ to the lesion was rate limiting for pol κ. It is therefore proposed that pol ζ and pol κ cooperate in lesion bypass, wherein pol ζ inserts the initial base(s) 3′ to the lesion followed by extension by pol κ to the point that normal replication can resume. For the N^2-IQ adduct, single knockdown of either pol η or ζ produced a similar reduction in MF, however, the double knockdown of both η and ζ produced a significant

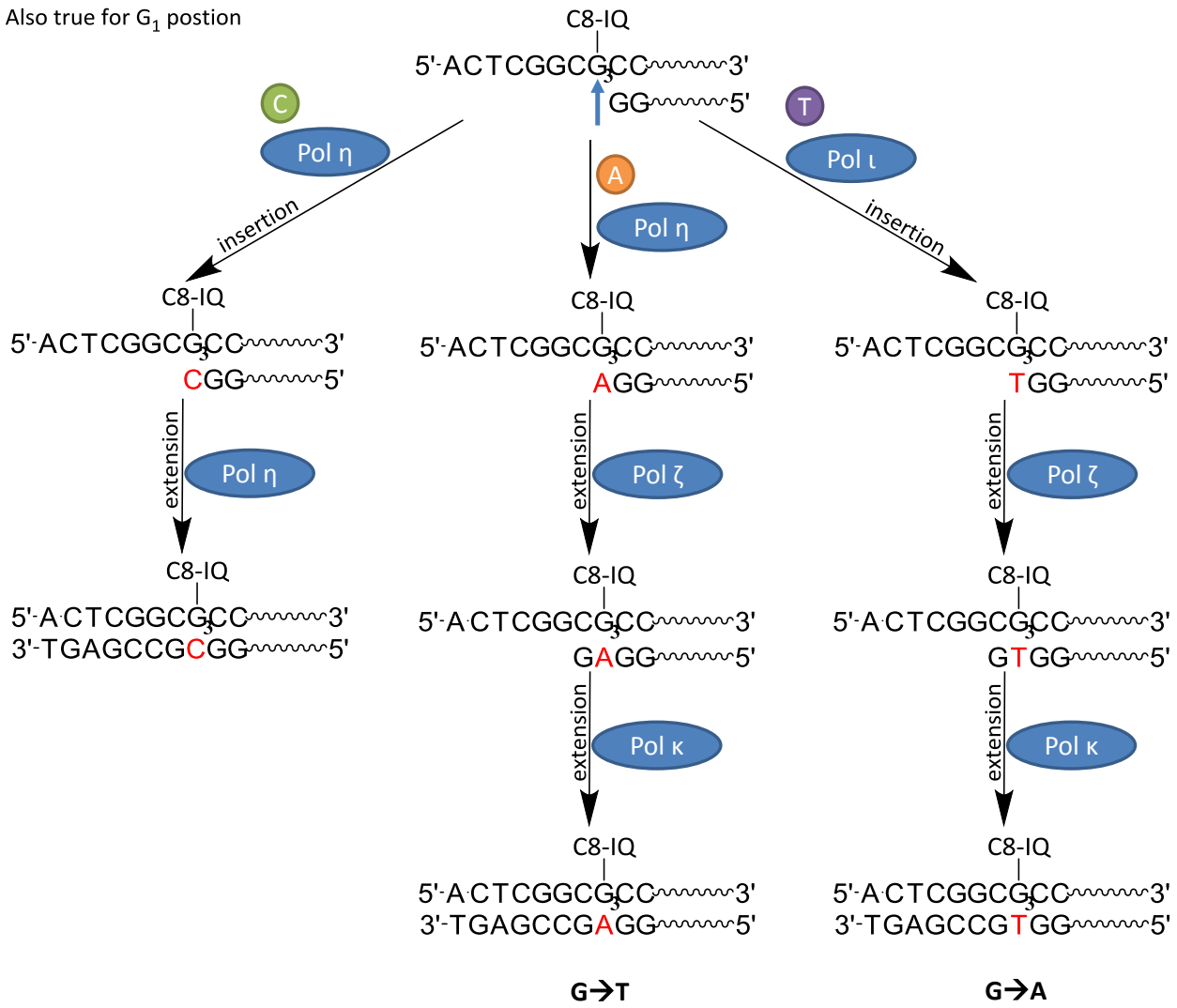

Figure 3-11: Summary primary TLS results for dG-C8-IQ.

reduction in MF. This indicates that both enzymes are responsible for the error-prone TLS of dG-N^2-IQ (**1.27**) and may cooperate in bypass of the lesion. Both pol η and ζ were observed to favor error-prone processing of dG-N^2-IQ (**1.27**) *in vitro.* In conclusion, our results indicate that the error-free bypass of IQ adducts is performed by pol η for dG-C8-IQ (**1.26**) and by pol κ for N^2-IQ (Figures 3-10 and 3-11). Our results also suggest pol κ and ζ are primarily responsible for error-prone TLS of dG-C8-IQ (**1.26**), while pol η and ζ are primarily responsible for error-prone TLS of dG-N^2-IQ (**1.27**) (Figure 3-10 and 3-11).

Experimental Procedures

Materials. yPol ζ, hPol κ, and hpol ι were purchased from Enzymax (Lexington, KY). hRev1 was either a gift from the laboratory of F. P. Geungerich (Vanderbilt University, Nashville, Tn) or purchased from Enzymax (Lexington, KY). The catalytic core of hPol η was a gift from the laboratory of Martin Egli at Vanderbilt University (Nashville, TN). The dNTP solutions (100 mM) were purchased from New England Biolabs (Ipswich, MA) or GE Healthcare (formerly Amersham Biosciences, Piscataway, NJ). [γ–^{32}P]ATP was purchased from Perkin Elmer (Waltham, MA). dG-N^2-IQ (**1.27**) modified oligonucleotides were prepared as previously reported.[6] Unmodified oligonucleotides were purchased from Midland Certified Reagents (Midland, TX).

Labeling and Annealing of Oligonucleotides. The primer was 5´ end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (New England Biolabs, Ipswich, MA) according to the manufacturer's instructions and purified on a Biospin column (BioRad, Hercules, CA). Each template and the respective ^{32}P-labeled primer (1:1 molar ratio) were annealed in Tris-HCl buffer

(50 mM, pH 7.5) by heating at 90 °C for 5 min and then slowly cooling to room temperature (r. t.) in accordance with previously published procedure.[6]

Single-Nucleotide Incorporation Assays. ^{32}P-labeled primers were annealed to either the unmodified or the dG-N^2-IQ (**1.27**) modified template, and extension reactions were then carried out in the presence of single dNTPs. All reactions were initiated by the addition of the dNTP solution (100 μM) to preincubated enzyme/DNA mixtures giving a final reaction volume of 20 μL. The final concentrations of the components for the incorporation assays were in Tris-HCl (50 mM, pH 7.5), DNA duplex (10 nM), ypol ζ (2.5, 5, or 10 nM) or Rev1 (2.5, 5, or 10 nM), dithiothreitol (DTT, 5 mM), bovine serum albumin (BSA, 50 μg/mL-1), NaCl (50 mM), and $MgCl_2$ (5 mM). The ypol ζ reactions were run at 37 °C for 2 hr. Reactions were quenched with equal volume of EDTA (20 μL, 20 mM) in 95% formamide (v/v) containing xylene cyanol and bromophenol blue dyes. Aliquots (20 μL) were separated by electrophoresis on a denaturing gel containing urea (8.0 M) and 16 % acrylamide (w/v) (from a 19:1 acrylamide/bisacrylamide solution, AccuGel, National Diagnostics, Atlanta, GA) with Tris borate buffer (80 mM, pH 7.8) containing EDTA (1 mM). The gel was exposed to a PhosphorImager screen (Imaging Screen K, Bio-Rad) overnight. The bands were visualized with a PhosphorImaging system (Bio-Rad, Molecular Imager FX) using the manufacturer's Quantity One software, version 4.3.0.

Full-Length Extension Assay with All Four dNTPs. The unmodified or dG-N^2-IQ (**1.27**) modified template was annealed to the ^{32}P-labeled 0-primers (with a 3'-C, A, or T) and extended in the presence of all four dNTPs (100 μM each) at 37 °C. Reaction times were 2hr for ypol ζ, 5 hr for pol κ, and 30 min for pol ι and the catalytic core of hpol η for 30min. Each reaction was initiated by adding the mixture of the dNTP solution to a preincubated enzyme/DNA mixtures in Tris-HCl (50 mM, pH 7.5) buffer containing DNA duplex (10 nM),

ypol ζ (2.5, 5.0, and 10.0 nM), hPol κ (0.2, 0.4, 1.0, 2, and 3.3 nM), hpol ι (0.2, 0.4, 1.0, 2, and 3.3 nM), or hpol η (0.2, 0.4, 1.0, 2, and 3.3 nM), DTT (5 mM), BSA (50 μg/mL-1), NaCl (50 mM), and $MgCl_2$ (5 mM), giving a final reaction volume of 20 μL. Reactions were quenched by the addition of equal volume of EDTA (20 mM) in 95% formamide (v/v) containing xylene cyanol and bromophenol blue dyes. Aliquots (20 μL) were separated by electrophoresis on a denaturing gel containing urea (8.0 M) and 16 % acrylamide (w/v) (from a 19:1 acrylamide/bisacrylamide solution, AccuGel, National Diagnostics, Atlanta, GA) with Tris borate buffer (80 mM, pH 7.8), containing EDTA (1 mM). Gels were exposed to a PhosphorImager screen (Imaging Screen K, Bio-Rad) overnight. The bands were visualized with a PhosphorImaging system (Bio-Rad, Molecular Imager FX) using the manufacturer's Quantity One software, version 4.3.0.

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CHAPTER IV

SUMMARY: REPLICATION OF C8- AND N^2-DEOXYGUANOSINE OF 2-AMINO-3-METHYLIMIDAZO[4,5-*f*]QUINOLINE (IQ) ADDUCT

Introduction

2-Amino-3-methylimidazo[4,5-*f*]quinoline (IQ) is carcinogenic in laboratory animals including non-human primates, and has been classified as "reasonably anticipated to be a human carcinogen" by the National Toxicology Program.[1] IQ is formed when meats are cooked at normal temperatures through the Maillard reaction. Exposure to IQ occurs through the ingestion of meats prepared at normal cook temperatures.[2,3] Ingested IQ is activated in the liver toward adduction at guanine sites of DNA.[4]

The Role of TLS Polymerases in Replication of dG-IQ Adducts

Bulky DNA adducts such as dG-C8-IQ (**1.26**) and dG-N^2-IQ (**1.27**) are known to block DNA replication. The C8 and N^2-IQ adducts of dG are a strong block to *in vitro* TLS by pol δ.[5] In addition, the carbocyclic anaolog of dG-C8-IQ (**1.26**) inhibited replication by *E. coli* DNA polymerase I, exo-free Klenow fragment, exo-free DNA polymerase II, and Dpo4.[6] Previously, human pol η was the only pol identified to efficiently insert opposite both dG-C8-IQ (**1.26**) and dG-N^2-IQ (**1.27**) and then extend the primers beyond dG-C8-IQ and dG-N^2-IQ (**1.26**) adduct sites *in vitro*. Pol κ and ι were shown to insert opposite the lesion dG-C8-IQ and -N^2-IQ lesions; however, further extension of primers was only observed from dG-N^2-IQ adduct site. [6]

In recent studies, TLS pols ζ, Rev1, κ, ι, and η are implicated in the TLS of the dG-N^2-IQ

and dG-C8-IQ lesions through siRNA knockdown of the TLS pols in HEK293T cells.[7,8] However, TLS was not wholly dependent upon any one polymerase, as was the case for the C8-dG adduct of 3-nitrobenzanthrone (dG-C8-ABA).[9] The TLS of the IQ lesions was examined at each guanine in the *Nar*I recognition sequence (5′-$G_1G_2CG_3CC$-3′), a hotspot for frameshift mutations.[8] However, the mutations induced in HEK293T cells for both dG-C8-IQ and -N^2-IQ were point mutations; no frameshift mutations were observed.[8] The majority of mutations observed were G→T transversions, although some G→A transitions were also seen. In these studies, the percentage of TLS in wildtype cells (absence of knockdown) transfected with dG-N^2-IQ was approximately half the percentage of TLS observed in HEK293T cells containing the dG-C8-IQ adduct (Figure 2-01 and 3-01). The low percentage of TLS observed for dG-N^2-IQ implies the lesion participates in a greater number of blocked replication events than dG-C8-IQ lesion. These blocked replication events could lead to strand breaks. The roles of the TLS pols ζ, Rev1, κ, ι, and η were revealed by evaluating the mutation frequencies induced by IQ lesions when TLS pols were knocked down in HEK 293T cells (Figure 2-02 and 3-02).[8] An increase in mutation frequency was observed for dG-C8-IQ (**1.26**) and dG-N^2-IQ (**1.27**) when pol η and pol κ were respectively knocked down (Figure 2-02 and 3-02). Therefore, the pol η plays a role in non-mutagenic bypass of dG-C8-IQ (**1.26**) and pol κ plays a role in non-mutagenic bypass of dG-N^2-IQ (**1.27**). The knockdown of the other TLS pols ζ, Rev1, κ, and ι for dG-C8-IQ (**1.26**) and pols ζ, Rev1, ι, and η for dG-N^2-IQ (**1.27**) resulted in a decrease in MF, indicating a role in mutagenic bypass (Figure 2-02 and 3-02). For the individual adduct dG-C8-IQ (**1.26**) or dG-N^2-IQ (**1.27**), the roles of the TLS polymerases in non-mutagenic or mutagenic bypass of the respective adduct were independent of the position of the adducted G in the *Nar*I site, even though the conformation of dG-C8-IQ changes depending on the position (Figure 2-02 and 3-

02).[10] The conformation of dG-C8-IQ is *syn* about the glycosidic bond at all positions, and intercalated at G_3 and minor groove bound at G_1 and G_2.[10] For comparison, the conformation of dG-N^2-IQ is *anti* about glycosidic bond and intercalated at G_1 and G_3.[11,12] The difference between the enzymes primarily responsible for non-mutagenic bypass of dG-C8-IQ (**1.26**) and dG-N^2-IQ (**1.27**) is significant. The literature available suggests the difference is related to the size of the adduct and whether the C8 or N^2 atom of dG is modified.[13,14] The suggested roles are that pol η has difficulty bypassing bulky N^2-guanine adducts, while pol κ is highly proficient at bypassing bulky N^2-guanine adducts. The mutation frequency observed for dG-C8-IQ (**1.26**) and dG-N^2-IQ (**1.27**) lesion were greatest at G_3 and G_1 of the *N*arI site, respectively (Figure 2-02 and 3-03). A study showing pol κ preferentially participates in bypass of bulky N^2-guanine adducts suggest the TLS polymerase participation in bypass maybe significantly impacted position of dG modification (C8 or N^2).[13] Our results where pol η has a role in non-mutagenic bypass of dG-C8-IQ (**1.26**) and pol κ has a role in non-mutagenic bypass of dG-N^2-IQ (**1.27**) support these findings.[8] The small discrepancies in mutation frequency observed for each adduct may be related to adduct conformation and local sequence.[10,11,12]

In cellular studies, dG-C8-IQ (**1.26**) and dG-N^2-IQ (**1.27**) induced primarily G→T transversions and a minor amount of G→A transitions in HEK293T cells; importantly, no frameshift mutations were observed.[8] As mentioned, siRNA knockdown studies suggest that pol η and κ, respectively are essential for the error-free bypass of the dG-C8-IQ (**1.26**) and dG-N^2-IQ (**1.27**) lesions in HEK293T cells. Double knockdown reduced MF to less than 5%for dG-C8-IQ (**1.26**) with pols κ/ζ and for dG-N^2-IQ (**1.27**) with pols η/ζ. These results indicate the enzymes pols κ/ζ and pols η/ζ are significant in mutagenic bypass of the respective lesions. Thus, pols ζ/κ and pols η/ζ are implicated in the generation of the observed base substitution mutations induced

by dG-C8-IQ (**1.26**) and dG-N^2-IQ (**1.27**) in HEK293T cells.[8] Pol η and ι were previously associated with these observed mutations through the observation that pol η will insert dATP and pol ι will insert dTTP opposite of the dG-C8-IQ (**1.26**) and dG-N^2-IQ (**1.27**) *in vitro*. However, when misinsertion occurred, pol η and ι did not extend from the misinserted bases.[5] Therefore, the polymerases responsible for extending the dG-C8-IQ (**1.26**) and dG-N^2-IQ (**1.27**) mispaired primer termini were unknown. The role of TLS polymerases ζ, Rev1, κ, ι, and η in contributing to the G→T transversions and G→A transitions were further examined through *in vitro* extension from the primer terminus dG-C8-IQ:N and dG-N^2-IQ:N, where N is C, A, or T at both the G_1 and the iterated G_3 position of the *Nar*I site.[8] Pol ζ and Rev1 were also evaluated for the ability to insert nucleotides opposite of the dG-IQ lesions. Pol ζ was incapable of insertion for both lesions. The lack of insertion by pol ζ is consistent with reports that pol ζ acts primarily as an extender.[15, 16] Rev1 was able to insert C opposite of dG-N^2-IQ (**1.26**) *in vitro*, but not the dG-C8-IQ (**1.27**) (Figure 2-04 and 3-04). Rev1 is suggested to favor insertion opposite N^2-guanine adducts.[17] The overall results of the *in vitro* and cellular studies implicated each of the polymerases investigated in the error-prone TLS of IQ adducts to some extent. For both adducts, dG-C8-IQ:N and dG-N^2-IQ:N, where N is C, A, or T, replication by pol ι was minor both *in vitro* and in cellular studies. Pol ι was observed to contribute to error-prone TLS primarily through the insertion of dTTP opposite of the lesion and through one base extension from dG_1-C8 and dG_1-N^2-IQ:A and dG_3-N^2-IQ:A (Figure 2-07 and 3-07). For the C8-IQ adduct, the reduction in MF upon double knockdown of pol κ and ζ indicated both pols were essential in error-prone TLS in HEK293T cells (Figure 2-02).[8] The results suggest that the polymerases cooperatively bypass the C8 lesion. The *in vitro* results indicate that the insertion of the first primer base 3′ to the lesion was rate limiting for pol κ (Figure 2-06).

It is therefore proposed that pol ζ and pol κ cooperate in lesion bypass, wherein pol ζ inserts the initial base(s) 3′ to the lesion followed by extension by pol κ to the point where replicative polymerases can resume (Figure 2-05 and 2-06).[8] For the N^2-IQ adduct, single knockdown of either pol η or ζ produced a similar reduction in MF; however, the double knockdown of both η and ζ produced a significant reduction in MF (Figure 3-02). This indicates that both enzymes are responsible for the error-prone TLS of dG-N^2-IQ (**1.27**) and may cooperate in bypass of the lesion. Both pol η and ζ were observed to favor error-prone processing of dG-N^2-IQ (**1.27**) *in vitro* (Figure 3-06 and Figure 3-08). In conclusion, our results indicate that the error-free bypass of IQ adducts is performed by pol η for dG-C8-IQ (**1.26**) and by pol κ for N^2-IQ (Figures 3-10 and 3-11).[8] Our results also suggest pol κ and ζ are primarily responsible for error-prone TLS of dG-C8-IQ (**1.26**), while pol η and ζ are primarily responsible for error-prone TLS of dG-N^2-IQ (**1.27**) (Figure 3-10 and 3-11).[8]

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