

# Translesion Synthesis of the $N^2$ -2'-Deoxyguanosine Adduct of the Dietary Mutagen IQ in Human Cells: Error-Free Replication by DNA Polymerase $\kappa$ and Mutagenic Bypass by DNA Polymerases $\eta$ , $\zeta$ , and Rev1

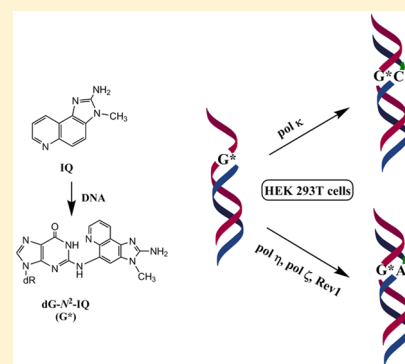
Arindam Bose,<sup>†,§</sup> Amy D. Millsap,<sup>‡</sup> Arnie DeLeon,<sup>‡,||</sup> Carmelo J. Rizzo,<sup>‡</sup> and Ashis K. Basu<sup>\*,†</sup>

<sup>†</sup>Department of Chemistry, University of Connecticut, Storrs, Connecticut 06269, United States

<sup>‡</sup>Department of Chemistry, Vanderbilt University, Nashville, Tennessee 37232, United States

**S** Supporting Information

**ABSTRACT:** Translesion synthesis (TLS) of the  $N^2$ -2'-deoxyguanosine (dG- $N^2$ -IQ) adduct of the carcinogen 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) was investigated in human embryonic kidney 293T cells by replicating plasmid constructs in which the adduct was individually placed at each guanine ( $G_1$ ,  $G_2$ , or  $G_3$ ) of the *NarI* sequence (5'-CG<sub>1</sub>G<sub>2</sub>CG<sub>3</sub>CC-3'). TLS efficiency was 38%, 29%, and 25% for the dG- $N^2$ -IQ located at  $G_1$ ,  $G_2$ , and  $G_3$ , respectively, which suggests that dG- $N^2$ -IQ is bypassed more efficiently by one or more DNA polymerases at  $G_1$  than at either  $G_2$  or  $G_3$ . TLS efficiency was decreased 8–35% in cells with knockdown of pol  $\eta$ , pol  $\kappa$ , pol  $\iota$ , pol  $\zeta$ , or Rev1. Up to 75% reduction in TLS occurred when pol  $\eta$ , pol  $\zeta$ , and Rev1 were simultaneously knocked down, suggesting that these three polymerases play important roles in dG- $N^2$ -IQ bypass. Mutation frequencies (MFs) of dG- $N^2$ -IQ at  $G_1$ ,  $G_2$ , and  $G_3$  were 23%, 17%, and 11%, respectively, exhibiting a completely reverse trend of the previously reported MF of the C8-dG adduct of IQ (dG-C8-IQ), which is most mutagenic at  $G_3$  ((2015) *Nucleic Acids Res.* 43, 8340–8351). The major type of mutation induced by dG- $N^2$ -IQ was targeted G  $\rightarrow$  T, as was reported for dG-C8-IQ. In each site, knockdown of pol  $\kappa$  resulted in an increase in MF, whereas MF was reduced when pol  $\eta$ , pol  $\iota$ , pol  $\zeta$ , or Rev1 was knocked down. The reduction in MF was most pronounced when pol  $\eta$ , pol  $\zeta$ , and Rev1 were simultaneously knocked down and especially when the adduct was located at  $G_3$ , where MF was reduced by 90%. We conclude that pol  $\kappa$  predominantly performs error-free TLS of the dG- $N^2$ -IQ adduct, whereas pols  $\eta$ , pol  $\zeta$ , and Rev1 cooperatively carry out the error-prone TLS. However, *in vitro* experiments using yeast pol  $\zeta$  and  $\kappa$  showed that the former was inefficient in full-length primer extension on dG- $N^2$ -IQ templates, whereas the latter was efficient in both error-free and error-prone extensions. We believe that the observed differences between the *in vitro* experiments using purified DNA polymerases, and the cellular results may arise from several factors including the crucial roles played by the accessory proteins in TLS.



## INTRODUCTION

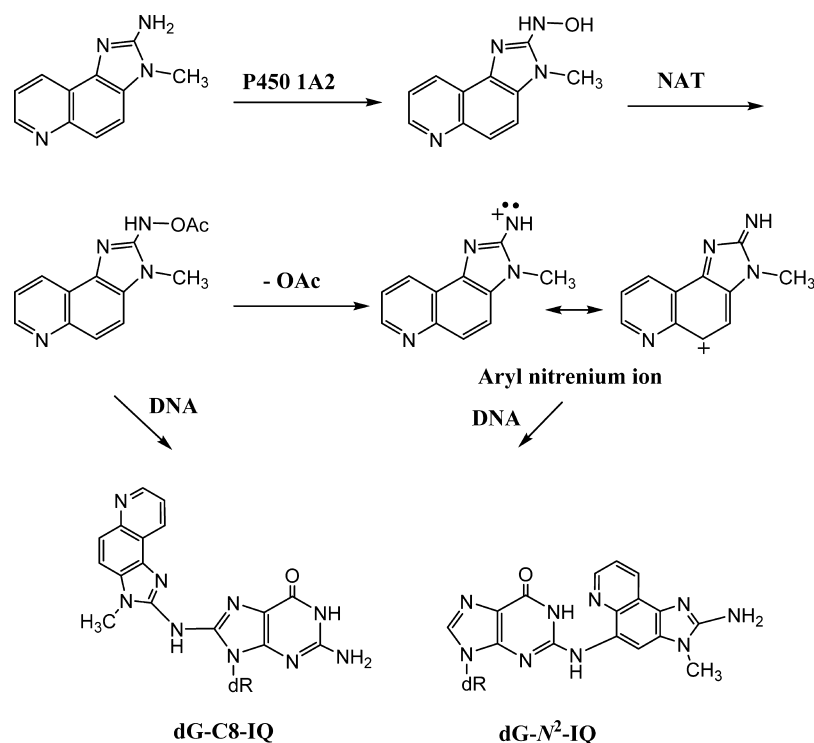
2-Amino-3-methylimidazo[4,5-*f*]quinoline (IQ), a heterocyclic amine found in cooked meat,<sup>1</sup> is generated by the Maillard reaction upon pyrolysis of reducing sugars and amino acids. Although the exposure level of IQ to humans is low (~60 ng/day),<sup>2</sup> it is believed to be involved in the development of human cancer.<sup>3</sup> In addition to cooked meats,<sup>4,5</sup> it is present in tobacco smoke.<sup>6</sup> IQ is a potent mutagen in Ames' *Salmonella typhimurium* assay and is "reasonably anticipated to be a human carcinogen" according to the National Toxicology Program.<sup>3,7,8</sup> While IQ induces tumors in various organs in rats, mice, and primates, it is principally a liver carcinogen.<sup>9–11</sup>

In cells, IQ must undergo bioactivation prior to DNA adduction.<sup>12</sup> IQ is converted to its *N*-hydroxylamine by cytochrome P450 1A2, which is acetylated by *N*-acetyltransferase, predominantly NAT2 (Figure 1).<sup>13,14</sup> The *N*-acetoxy-IQ (or the nitrenium ion formed from it) is the ultimate carcinogen, that forms DNA adducts (Figure 1).<sup>15</sup> Metabolic activation of IQ also causes oxidative DNA damage,

but this process is unlikely to play a role in IQ carcinogenesis.<sup>16</sup> Metabolically activated IQ generates a main DNA adduct (dG-C8-IQ) at the C8-position of 2'-deoxyguanosine (dG) as well as a less abundant  $N^2$ -dG adduct (dG- $N^2$ -IQ) (Figure 1).<sup>17–19</sup> However, the latter is slowly repaired and persists for a long time in rodents.<sup>20</sup> In Ames' test, IQ induces high levels of dinucleotide deletions in the CpG repeat sequences of the *HisD3052* target sequence (5'-CGCGCGCG-3'), which is common for many aromatic amines and nitroaromatic compounds.<sup>8,21</sup> Single adduct mutagenesis studies showed that the C8-dG adducts derived from metabolically activated 1-nitropyrene, and 1,6- and 1,8-dinitropyrenes cause two-base deletions in repetitive CpG sequences in bacteria, whereas mostly base substitutions occur in simian kidney cells.<sup>22–24</sup> Like the CpG repeat sequence, the *NarI* restriction site 5'-CG<sub>1</sub>G<sub>2</sub>CG<sub>3</sub>CC-3' is a hot-spot for frameshift mutagenesis for

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**Figure 1.** Metabolic activation and the DNA adduct formation by IQ.

the C8-dG adduct of *N*-acetyl-2-aminofluorene, primarily when the adduct is positioned at G<sub>3</sub>, but only base substitutions occur in simian kidney cells.<sup>25–27</sup> The diversity in the types and frequencies of mutations caused by the DNA lesions in different cells and organisms may stem from the differences in DNA polymerases (pols) that bypass them.<sup>28</sup> Bulky DNA adducts such as the ones formed by IQ are usually strong blocks of replicative DNA pils, but a group of specialized translesion synthesis (TLS) DNA pils can bypass them.<sup>29–32</sup> In eukaryotic cells, TLS is carried out by the Y-family pils, pol  $\eta$ , pol  $\kappa$ , pol  $\iota$ , and Rev1 and the B-family enzyme pol  $\zeta$ .<sup>32,33</sup> The TLS pils are characterized by high error rates on undamaged templates, although some of them are equipped to bypass specific DNA damages efficiently and with high fidelity.<sup>34,35</sup> In eukaryotic cells, often efficient TLS involves two sequential steps.<sup>36,37</sup> In the first step, the stalled replicative pil is replaced by a TLS pil, which inserts a nucleotide opposite the DNA lesion. In the subsequent step, this inserter pil may continue with the lesion bypass or be substituted by another TLS pil for extension of the primer a few nucleotides past the lesion site. This TLS pil is eventually replaced by the replicative pil to restart and continue DNA synthesis. In a cell, accessory proteins, such as proliferating cell nuclear antigen (PCNA) that is activated by monoubiquitination after DNA damage, are also required for efficient TLS.<sup>38,39</sup>

*In vitro* studies established that pol  $\delta$ , a replicative B-family pil, cannot bypass the IQ adducts.<sup>40</sup> In the *NarI* restriction site, human pol  $\eta$  (hpol  $\eta$ ) is able to extend primers beyond both dG-C8-IQ and dG-N<sup>2</sup>-IQ more efficiently than pol  $\kappa$  and much more efficiently than pol  $\iota$  and pol  $\delta$ .<sup>40</sup> TLS by pol  $\eta$  is largely error-free for dG-C8-IQ. A two-base deletion occurred with the dG-N<sup>2</sup>-IQ adduct at the G<sub>3</sub> site, while the product was error-free when the adduct was located at G<sub>1</sub> but pol  $\eta$  failed to carry out further extension. In a cell, however, TLS is far more complex, owing to the involvement of multiple proteins, and

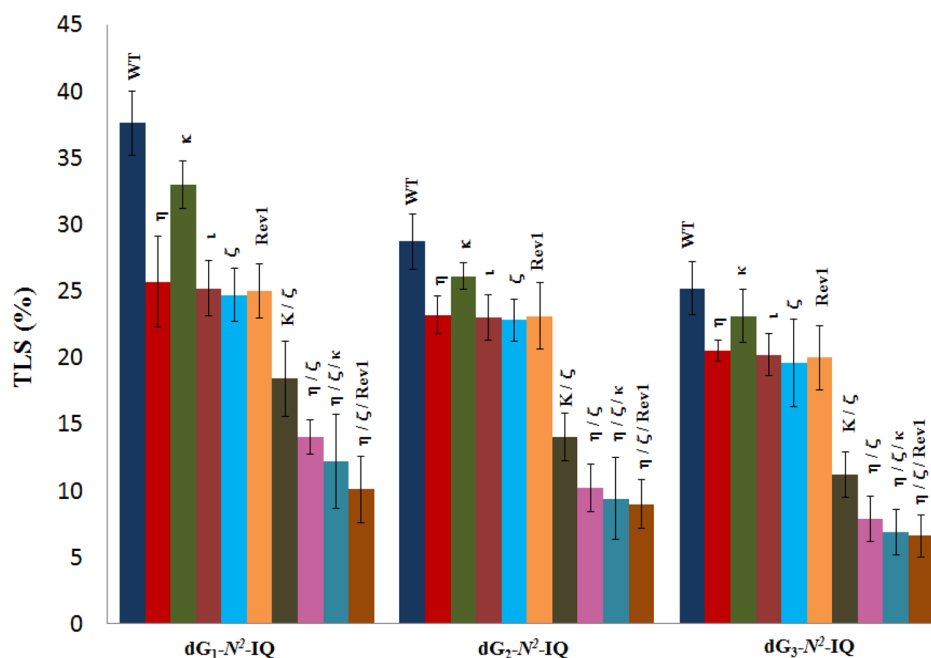
the mode of selection and recruitment of the TLS pil that bypasses the DNA lesion has not yet been established.<sup>41</sup> In a recent work, we found that most of the error-prone TLS of dG-C8-IQ is performed by pol  $\kappa$  and pol  $\zeta$ , whereas pol  $\eta$  carries out error-free bypass in human cells.<sup>42</sup> We also determined that both TLS and the mutation frequency (MF) of dG-C8-IQ situated at the three guanines of the *NarI* site are significantly different.<sup>42</sup> In the current investigation, using an approach similar to the prior study of dG-C8-IQ, we have explored the roles of different TLS pils in bypassing dG-N<sup>2</sup>-IQ located in the three different guanine sites of the *NarI* restriction sequence. We report herein that dG-N<sup>2</sup>-IQ is mutagenic in human embryonic kidney (HEK) 293T cells and induces mainly G  $\rightarrow$  T transversions but the MF is different at the three guanine sites. We also show that pol  $\kappa$  is more efficient in its TLS than the other pils and that it performs TLS principally in an error-free manner. In contrast, pol  $\eta$ , pol  $\zeta$ , and Rev1 cooperatively perform the majority of the mutagenic bypass.

## EXPERIMENTAL PROCEDURES

**Materials.** Yeast pol  $\zeta$ , human pol  $\kappa$ , and Rev1 were obtained from Enzymax (Lexington, KY). The dNTP solutions (100 mM) were from New England Biolabs (Ipswich, MA) or GE Healthcare (Piscataway, NJ). [ $\gamma$ -<sup>32</sup>P]ATP was obtained from PerkinElmer (Waltham, MA). dG-N<sup>2</sup>-IQ modified oligonucleotides were synthesized according to a literature procedure.<sup>40</sup> Unmodified oligonucleotides were from Midland Certified Reagents (Midland, TX).

**siRNAs.** Synthetic siRNA duplexes against *POLH*, *POLK*, *POLL*, *REV1* and negative control (NC) siRNA were from Qiagen (Valencia, CA). siRNA for *REV3* was acquired from Integrated DNA Technologies (Coralville, IA). Sequences of all the siRNAs are as reported in ref 43.

**Methods.** *Construction and Characterization of dG-N<sup>2</sup>-IQ-Containing Plasmids and Their Replication in HEK293T Cells.* Site-specific adduct-containing pMS2 vectors with neomycin and ampicillin resistance genes were constructed in a manner similar to the construction of the dG-C8-IQ-containing vectors.<sup>42</sup> Transfection of



**Figure 2.** Effects of siRNA knockdowns of TLS pols on the extent of replicative bypass of dG- $N^2$ -IQ. Percent TLS in various pol knockdowns was measured relative to an internal control in which a different 12-mer oligonucleotide was inserted (i.e., 5'-GTGCGTGTGGT-3' in place of 5'-CTCG<sub>1</sub>G<sub>2</sub>CG<sub>3</sub>CCATC-3') into the gapped plasmid in a manner similar to the construction of the dG- $N^2$ -IQ (or control) construct. The data represents the mean of results from two independent experiments. HEK293T cells are treated with negative control (NC) siRNA, whereas the other single, double, and triple pol(s) knockdowns are indicated above the bar.

50 ng of each construct was performed in HEK293T cells, after they were grown to ~90% confluency, using 6  $\mu$ L of Lipofectamine cationic lipid reagent (Invitrogen, Carlsbad, CA). Subsequently, the cells were grown at 37 °C in 5% CO<sub>2</sub> for 48 h, and the plasmid DNA was isolated and purified.<sup>44</sup> It was used to transform *E. coli* DH10B, and the progeny were examined as described.<sup>24,45</sup>

**Determination of TLS Efficiency.** Single-stranded pMS2 DNA construct containing a different DNA sequence where the 12-mer oligonucleotide was inserted (i.e., 5'-GTGCGTGTGGT-3' in place of 5'-CTCG<sub>1</sub>G<sub>2</sub>CG<sub>3</sub>CCATC-3') into the gapped plasmid was mixed with the adducted or control constructs (1:1). The DNA mixture was used to transfect HEK293T cells and processed as described above. Oligonucleotide probes for both the wild type and the mutant plasmid were used to analyze the progeny. The unmodified DNA was used as an internal control so that TLS efficiency could be determined from the percentages of the colonies originating from the adduct-containing plasmid relative to that from the unmodified plasmid.

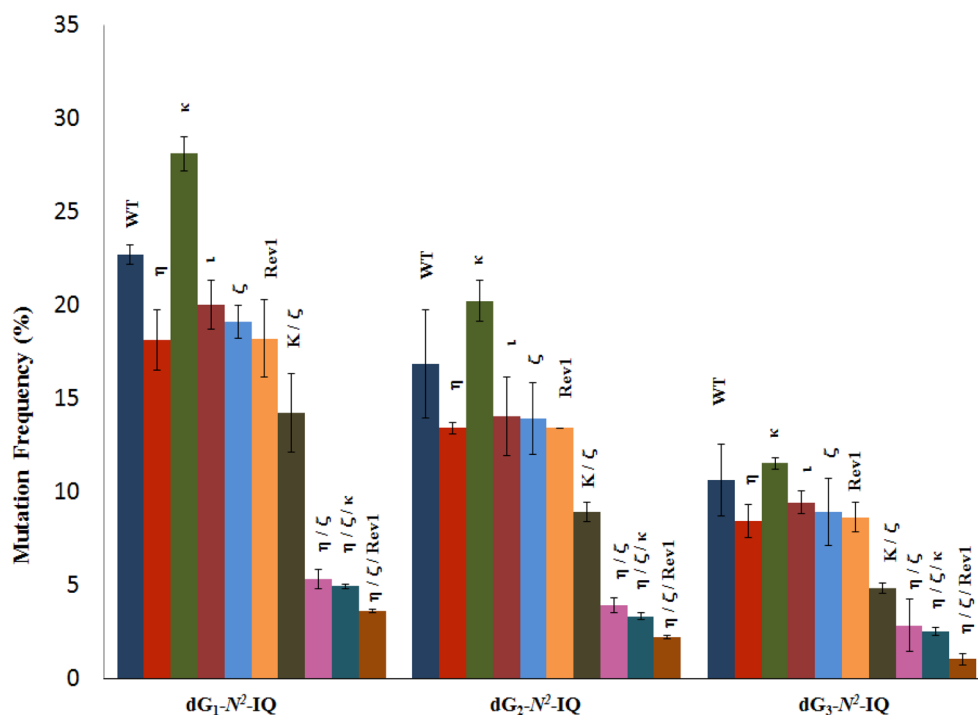
**Mutational Analyses of TLS Products from Human Cells with Pol Knockdowns.** HEK293T cells were transfected with synthetic siRNA duplexes and plated in 6-well plates at 50% confluence. Following a 24-h incubation, they were seeded in 24-well plates at 70% confluence a day before transfection of the plasmid. Subsequently, cells were cotransfected with another aliquot of siRNA and the adducted or control plasmid. Following a 24-h incubation, plasmid DNA was isolated and used to transform *E. coli* as described above. RT-PCR and Western blotting were performed as reported.<sup>43</sup> Mutations were determined by oligonucleotide hybridization followed by DNA sequence analyses around the *NarI* site. Two 15-mer probes, complementary to part of the inserted oligonucleotide sequence on the left or right side of the plasmid where ligation took place and a part of the plasmid, were used to select plasmids containing the correct insert, and transformants that did not hybridize with both the left and right probes were omitted. A third probe containing the complementary 14-mer wild type sequence encompassing the entire inserted DNA sequence was used to analyze the progeny plasmids. Any clone that failed to hybridize with this probe was considered a putative mutant and subjected to DNA sequencing.

**Single-Nucleotide Incorporation Assays by hRev1.** <sup>32</sup>P-labeling, annealing, and extension reactions of the primers on the unmodified or the dG- $N^2$ -IQ modified template by Rev1 were carried out in the presence of dCTPs in a manner similar to that in ref 42. Details are provided in the SI.

**Full-Length Extension Assay by yPol  $\zeta$  or Pol  $\kappa$  with All Four dNTPs.** Annealing the <sup>32</sup>P-labeled 0-primers (with a 3'-C or A) to the unmodified or dG- $N^2$ -IQ modified template and extension in the presence of all four dNTPs (100  $\mu$ M each) at 37 °C for 5 h followed the method reported earlier.<sup>42</sup> Details are provided in the SI.

## RESULTS

**Roles of Pol  $\eta$ ,  $\kappa$ , and  $\zeta$  in TLS of dG- $N^2$ -IQ.** The construction of the adduct containing vector, its replication in human cells, and progeny analysis followed an approach described earlier.<sup>24,42,46</sup> In order to identify the pols involved in TLS of dG- $N^2$ -IQ in human cells, the siRNA knockdown approach was used to limit the expression of specific TLS pol(s) in HEK293T cells.<sup>43</sup> An internal control plasmid, containing an undamaged 12-mer of different sequence in place of the 12-mer adducted oligonucleotide, was mixed with the adduct-containing plasmid before transfection. TLS efficiency was calculated by the percentages of the colonies originating from each dG- $N^2$ -IQ-containing plasmid relative to that from the unmodified internal control. In HEK293T cells, the frequency of TLS was 38%, 29%, and 25% for the dG- $N^2$ -IQ placed at G<sub>1</sub>, G<sub>2</sub>, and G<sub>3</sub>, respectively, relative to 100% progeny derived from the undamaged plasmid construct (Figure 2). This suggests that dG- $N^2$ -IQ is bypassed considerably more efficiently by one or more pols at G<sub>1</sub> than at either G<sub>2</sub> or G<sub>3</sub>. TLS efficiency was decreased in cells with knockdown of each TLS pol. Only 8–12% reduction in TLS efficiency was observed in cells with knockdown of pol  $\kappa$ , whereas knockdown of pol  $\eta$ , pol  $\iota$ , pol  $\zeta$ , or Rev1 resulted in 20–35% reduction in TLS. In the pol knockdown experiments, the location of the



**Figure 3.** Mutational frequency of dG- $N^2$ -IQ derived from the progeny from G<sub>1</sub>, G<sub>2</sub>, and G<sub>3</sub> constructs in HEK293T cells, also transfected with NC siRNA (WT) or siRNA for single, double, or triple pol(s) knockdowns, is shown. The data represent the average of two independent experiments (presented in Table S1A–J in SI).

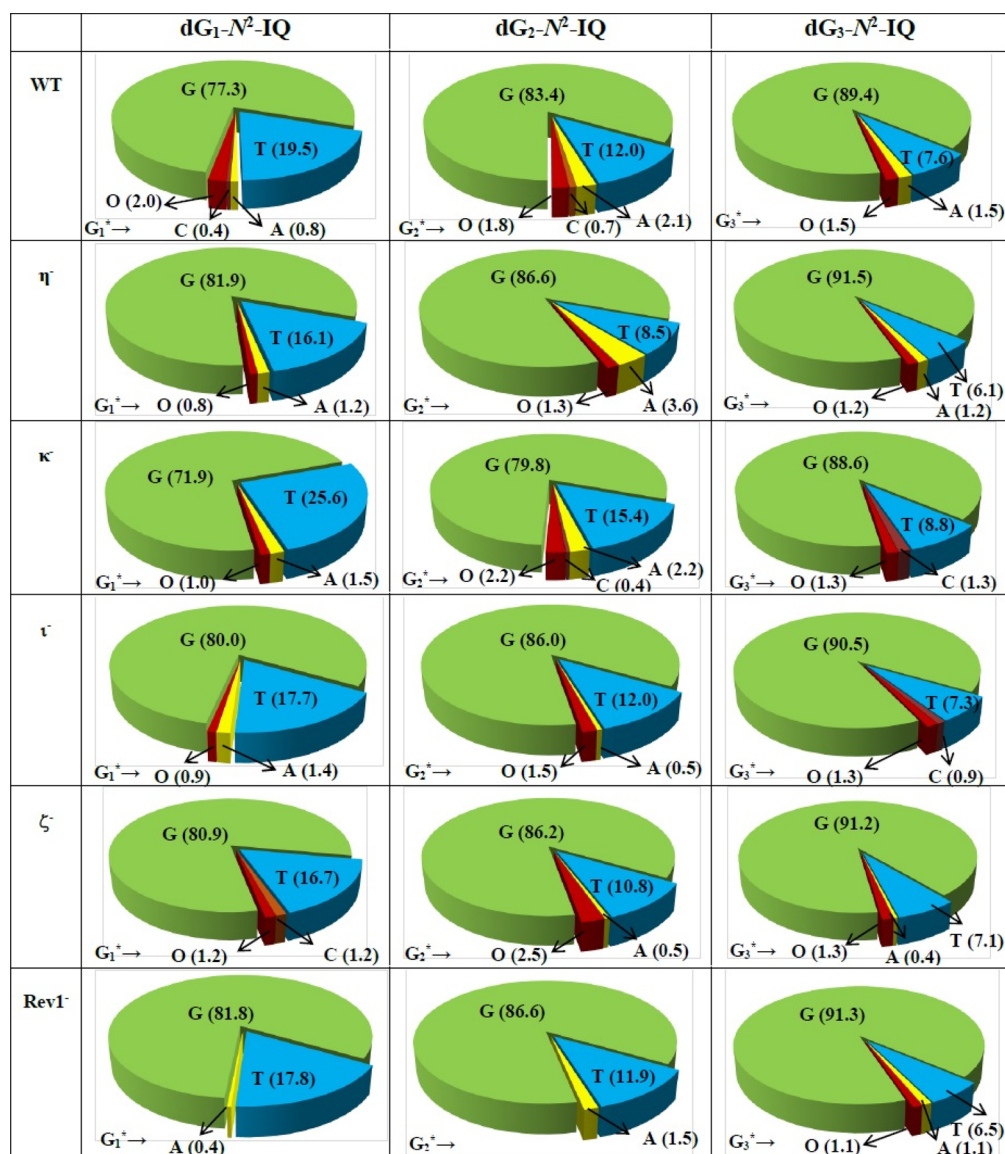
adduct did not significantly influence the TLS efficiency. A more prominent effect on TLS was observed upon simultaneous knockdown of pol  $\eta$ /pol  $\zeta$  and pol  $\kappa$ /pol  $\zeta$  (Figure 2). Simultaneous knockdown of pol  $\kappa$  and pol  $\zeta$  resulted in approximately 50% reduction in TLS, but simultaneous knockdown of pol  $\eta$ /pol  $\zeta$  showed a more noticeable outcome, resulting in up to 70% reduction in TLS when dG- $N^2$ -IQ was located at G<sub>3</sub>. The most significant reduction (~75%) in TLS occurred when pol  $\eta$ , pol  $\zeta$ , and Rev1 were simultaneously knocked down. We conclude that each TLS pol takes part in TLS of dG- $N^2$ -IQ and that pol  $\eta$ , pol  $\zeta$ , and Rev1 play complementary roles in TLS of dG- $N^2$ -IQ.

**Error-Free and Error-Prone Bypass of dG- $N^2$ -IQ in HEK293T Cells.** DNA sequence analysis showed that dG- $N^2$ -IQ is mutagenic in HEK293T cells in each of the three sites (Figure 3). As in the case of dG-C8-IQ, MFs were different at the three guanines, but they exhibited a completely reverse trend here. Unlike the dG-C8-IQ, in which the adduct at G<sub>3</sub> is most mutagenic, MF of dG- $N^2$ -IQ at G<sub>1</sub> (23%) was higher than that at G<sub>2</sub> (17%), which, in turn, was higher than the adduct located at G<sub>3</sub> (11%). However, the major types of mutations for dG-C8-IQ and dG- $N^2$ -IQ were the same in that they both induced targeted G  $\rightarrow$  T transversions (Figures 4 and 5). In each site, knockdown of pol  $\kappa$  resulted in an increase in MF, whereas MF was reduced when pol  $\eta$ , pol  $\iota$ , pol  $\zeta$ , or Rev1 was knocked down (Figure 3). This suggests that pol  $\kappa$  is involved in a greater fraction of error-free bypass of dG- $N^2$ -IQ, whereas pol  $\eta$ , pol  $\iota$ , pol  $\zeta$ , and Rev1 each participate in the error-prone TLS of this adduct. Simultaneous knockdown of two and three pols resulted in further reduction in MF. The diminution in MF was most pronounced when pol  $\eta$ , pol  $\zeta$ , and Rev1 were simultaneously knocked down and especially when the adduct was located at G<sub>3</sub> (Figures 3 and 5), where 90% reduction in MF was observed. We conclude that the error-prone TLS of the

dG- $N^2$ -IQ adduct occurs cooperatively by pols  $\eta$ , pol  $\zeta$ , and Rev1.

**In Vitro TLS of dG- $N^2$ -IQ by Eukaryotic Pols.** The *in vitro* replication of dG- $N^2$ -IQ at the iterated G<sub>3</sub>- and noniterated G<sub>1</sub>-positions of the *NarI* recognition sequence has been previously examined with purified human TLS pols  $\eta$ ,  $\kappa$ , and  $\iota$ , which showed that hpol  $\eta$  is the most efficient in bypassing both dG-C8-IQ and dG- $N^2$ -IQ.<sup>40</sup> Now, we also examined hRev1, which was reasonably efficient at inserting dCTP opposite the lesions (Figure S1 of the SI). In contrast, ypol  $\zeta$  was unable to insert a nucleotide opposite the dG- $N^2$ -IQ in either sequence context. Interestingly, the efficiency of hRev1 was higher when the modification was at the G<sub>1</sub>-position than for G<sub>3</sub> (Figure S1 of the SI). These results suggest that Rev1 could participate in nonmutagenic TLS. Further, the higher efficiency for insertion at G<sub>1</sub> suggested a more prominent role in nonmutagenic bypass at this position.

In previous studies, steady-state kinetic analysis showed a significant sequence context effect for the misinsertion of dATP opposite the dG- $N^2$ -IQ adduct by hpol  $\eta$ .<sup>40</sup> The misinsertion frequency for dATP opposite dG- $N^2$ -IQ was 0.042 and 0.71 when the lesion was situated at G<sub>3</sub>- and G<sub>1</sub>-positions, respectively. Cellular data showing that more G  $\rightarrow$  T mutations occurred at G<sub>1</sub> relative to G<sub>3</sub> is consistent with the *in vitro* kinetic result. However, only replication products from initial insertion of dCTP opposite the lesion were observed by mass spectrometry, suggesting that hpol  $\eta$  is inefficient in extending from an A opposite the dG- $N^2$ -IQ adduct.<sup>40</sup> Pol  $\zeta$  is typically implicated as an extender from the adduct:N template primer terminus. We, therefore, examined the ability of ypol  $\zeta$  to extend the dG- $N^2$ -IQ adduct when paired with C or mispaired with A. We observed that ypol  $\zeta$  was able to extend the primer by only one nucleotide in both sequence contexts (Figure 6). The one nucleotide extension appeared to be more efficient for



**Figure 4.** Types and frequencies of mutations induced by dG-N<sup>2</sup>-IQ in the progeny from the G<sub>1</sub>, G<sub>2</sub>, and G<sub>3</sub> constructs in HEK293T cells also transfected with NC siRNA (293T) or siRNA for single pol knockdowns are shown in a pie chart. O represents other (i.e. semi-targeted) mutations. The data represent the average of two independent experiments (presented in Table S1A–F in the SI).

the dG-N<sup>2</sup>-IQ:A mispair, particularly for the G<sub>3</sub>-modified template. In contrast, hpol  $\kappa$  was efficient at extending from the A opposite the dG-N<sup>2</sup>-IQ adduct in both sequence contexts (Figure 7). This suggests that pol  $\kappa$  may play a role in mutagenic bypass of the dG-N<sup>2</sup>-IQ adduct by extending from a mispaired A opposite the lesion. This would be in addition to its primary role in error-free replication of the lesion.

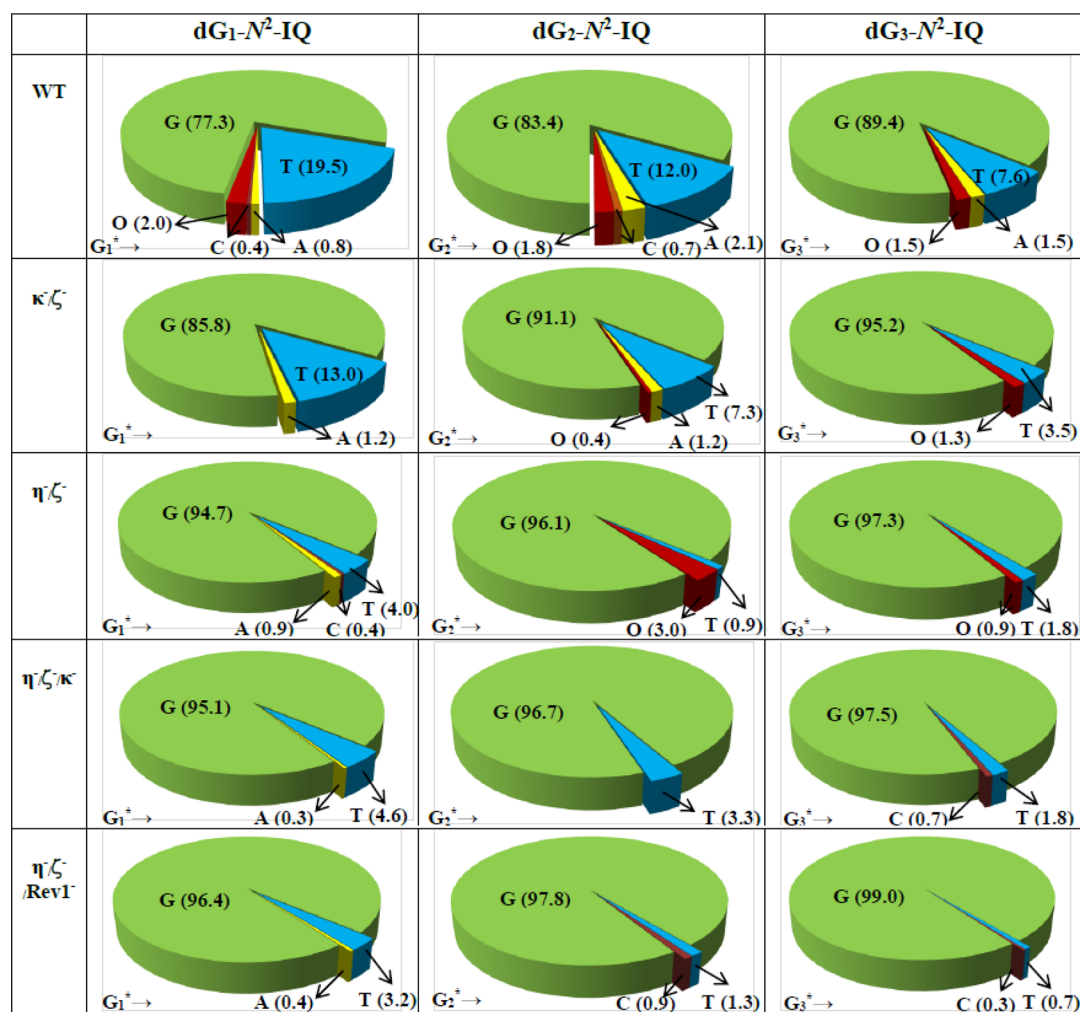
## DISCUSSION

**TLS Pols Active in Bypassing dG-N<sup>2</sup>-IQ.** Like many other bulky DNA adducts, both dG-C8-IQ and dG-N<sup>2</sup>-IQ are strong blocks of replication *in vitro*.<sup>47</sup> Pol  $\delta$  was completely inhibited by these adducts, whereas hpol  $\eta$  could extend primers beyond the adduct site more proficiently than hpol  $\kappa$  and significantly more efficiently than hpol  $\iota$  for each adducted G in the NarI sequence.<sup>40</sup>

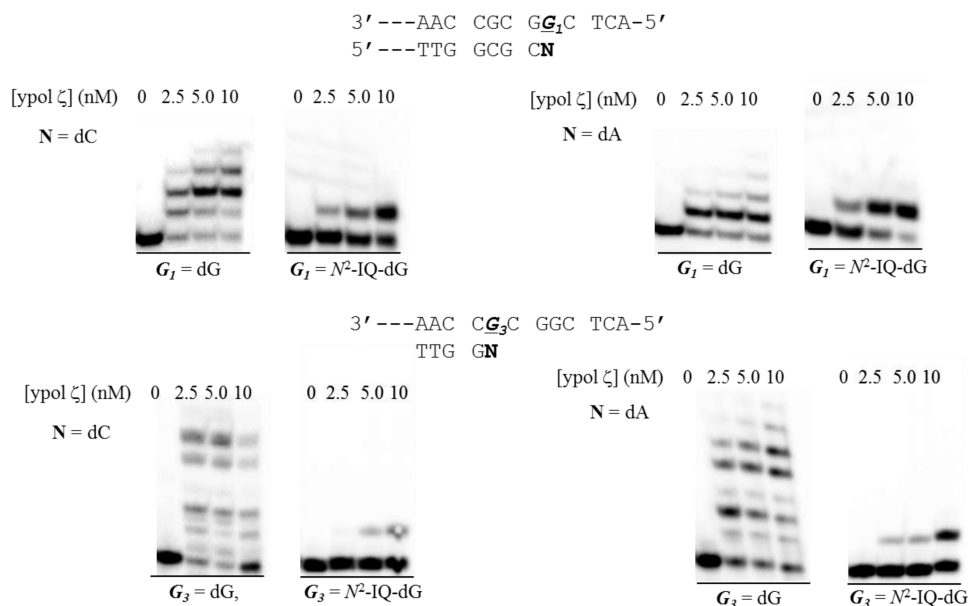
As reported earlier with the dG-C8-IQ, our current study suggests that each TLS pol we examined, including pol  $\eta$ , pol  $\kappa$ , pol  $\iota$ , and Rev1 of the Y-family and pol  $\zeta$  of the B-family, has a

role in TLS of dG-N<sup>2</sup>-IQ (Figure 2). Nevertheless, as with other bulky DNA adducts,<sup>43</sup> none of the TLS pols was indispensable for TLS of dG-N<sup>2</sup>-IQ. Knockdown of multiple pols resulted in a more marked effect on TLS. For example, concurrent knockdown of pol  $\eta$ , pol  $\zeta$ , and Rev1 reduced the TLS by ~75%, suggesting that they work cooperatively.

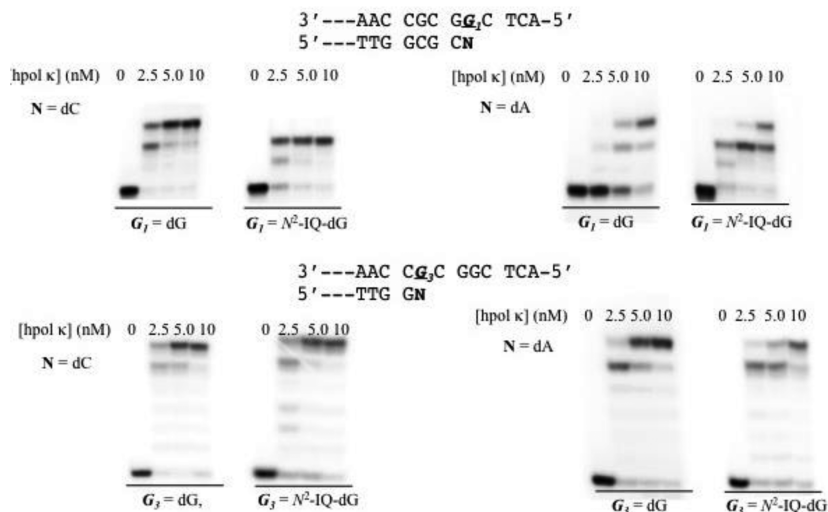
The results also establish that the magnitudes of the TLS of dG-N<sup>2</sup>-IQ differ at different sequence contexts (Figure 2). The major conformations of dG-N<sup>2</sup>-IQ in the three different sequences were found to be similar, adopting an intercalated conformation as determined when positioned at G<sub>1</sub> and G<sub>3</sub> of the NarI sequence.<sup>48,49</sup> However, these structures were determined on a fully duplex DNA, which may not be reflective of the structure at a replication fork bound to a pol. TLS pols have spacious active sites that can accommodate various conformation of the DNA adduct, and subtle conformational differences of the DNA adduct may influence their ability to bypass. Thus, the context effect of TLS likely relates to a small difference in the conformation of the DNA adduct. It is



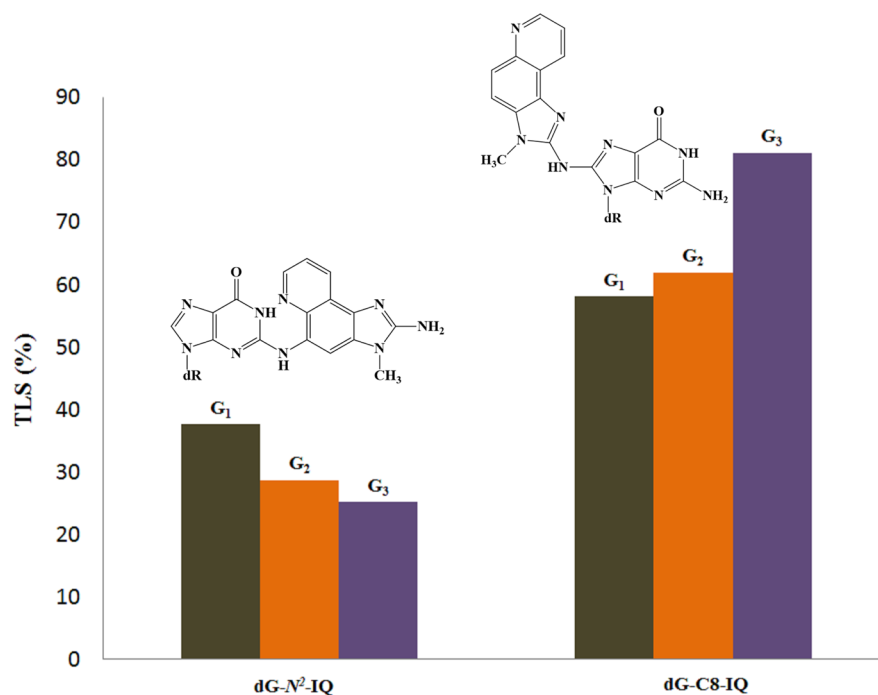
**Figure 5.** Types and frequencies of mutations induced by dG-N<sup>2</sup>-IQ in the progeny from the G<sub>1</sub>, G<sub>2</sub>, and G<sub>3</sub> constructs in HEK293T cells also transfected with NC siRNA (293T) or siRNA for double or triple pol(s) knockdowns are shown in a pie chart. O represents other (i.e. semi-targeted) mutations. The data represent the average of two independent experiments (presented in Table S1G–J in the SI).



**Figure 6.** Yeast pol ζ extension of a control and dG-N<sup>2</sup>-IQ modified template at the G<sub>1</sub> (top) or G<sub>3</sub> (bottom) positions when paired by dC (left) or mispaired with dA (right). Reactions were run with 10 nM DNA, 100 μM dNTPs, and 0, 2.5, 5.0, and 10 nM of ypol ζ.



**Figure 7.** Human pol  $\kappa$  extension of a control and dG- $N^2$ -IQ modified template at the G<sub>1</sub> (top) or G<sub>3</sub> (bottom) positions when paired by dC (left) or mispaired with dA (right). Reactions were run with 10 nM DNA, 100  $\mu$ M dNTPs, and 0, 2.5, 5.0, and 10 nM of hpol  $\kappa$ .

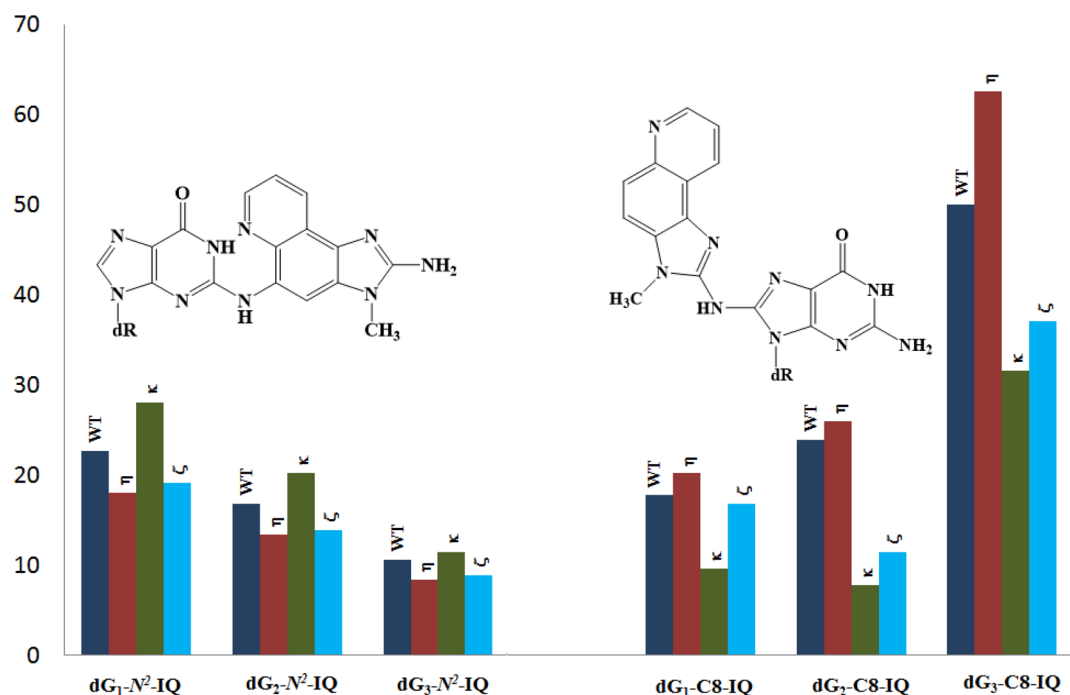


**Figure 8.** Comparison of TLS of dG- $N^2$ -IQ and dG-C8-IQ.

noteworthy that for dG- $N^2$ -IQ, the efficiency of TLS when the adduct was positioned at G<sub>1</sub> was higher than that at G<sub>2</sub>, which, in turn, was higher than that when it was located at G<sub>3</sub>. This pattern was exactly opposite of what was observed with dG-C8-IQ.<sup>42</sup>

**Error-Free versus Error-Prone TLS.** Similar to the TLS result, MFs of dG- $N^2$ -IQ were different in the three sites, and it was most mutagenic when positioned at G<sub>1</sub>. Of the several single pol knockdown experiments, an increase in MF was seen in pol  $\kappa$ -knockdown cells, whereas the reduction in MF was approximately the same upon knockdown of pol  $\eta$ , pol  $\iota$ , pol  $\zeta$ , or Rev1 (Figures 3 and 4). Error-free TLS, therefore, is largely carried out by pol  $\kappa$ , as reported for many other dG- $N^2$  adducts.<sup>40,50–52</sup> However, pol  $\eta$  and pol  $\zeta$  were found to be the most critical pols involved in erroneous TLS, as

demonstrated by the greater synergy in lowering MF upon simultaneous knockdown of these two pols (Figures 3 and 5). A previous *in vitro* study using a single pol, the steady-state kinetic insertion frequency of A relative to C opposite dG- $N^2$ -IQ by hpol  $\eta$  at G<sub>1</sub> was 71%, whereas the same at G<sub>3</sub> was only 4%, suggesting that hpol  $\eta$  is more error-prone at G<sub>1</sub>,<sup>40</sup> as observed in our current work. However, the extension of the dG- $N^2$ -IQ:A pair was inefficient by hpol  $\eta$ . Since pol  $\zeta$  was reported to extend efficiently after a nucleotide has been inserted opposite a DNA lesion,<sup>53–55</sup> based on this *in vitro* report and our current result, we postulate that pol  $\eta$  misincorporates dATP opposite dG- $N^2$ -IQ and the mismatch is extended by pol  $\zeta$ . Rev1 also has a role since further decrease in MF was noted when pol  $\eta$ , pol  $\zeta$ , and Rev1 were simultaneously knocked down. Unfortunately, we were unable to validate the



**Figure 9.** Comparison of MF of dG- $N^2$ -IQ and dG-C8-IQ derived from the progeny from G<sub>1</sub>, G<sub>2</sub>, and G<sub>3</sub> constructs in HEK293T cells, also transfected with NC siRNA (WT) or siRNA for single knockdowns of pol  $\eta$ , pol  $\kappa$ , or pol  $\zeta$ .

cellular results by *in vitro* experiments using ypol  $\zeta$ , which was only able to carry out one base extension of the dG- $N^2$ -IQ:C and dG- $N^2$ -IQ:A pair at both G<sub>1</sub> and G<sub>3</sub> sites (Figure 6). In fact, hpol  $\kappa$  was more efficient in extension than ypol  $\zeta$ . However, the ypol  $\zeta$  we employed contained the catalytic subunit Rev3 and the accessory subunit Rev7 only, whereas Pol31 and Pol32 were reported to be indispensable for pol  $\zeta$  function in TLS in yeast cells.<sup>56</sup> Furthermore, despite sequence homologies, the mammalian Rev3 is twice as large as its yeast homologue. Therefore, one can anticipate substantial differences in TLS of dG- $N^2$ -IQ by the four subunit (Rev3, Rev7, PolD2, and PolD3) human pol  $\zeta$  relative to the yeast enzyme.<sup>57</sup>

A comparison of the TLS of dG- $N^2$ -IQ with dG-C8-IQ is shown in Figure 8, demonstrating an opposite trend at the three sites by these two DNA adducts formed by the same carcinogen. Likewise, Figure 9 compares the MF of these two adducts located at G<sub>1</sub>, G<sub>2</sub>, and G<sub>3</sub>, which also exhibits a reverse trend. While an explanation for these trends must await future structural experiments, one particular noteworthy aspect in Figure 9 is that the role of pol  $\eta$  and pol  $\kappa$  were switched in the TLS of these two adducts. Pol  $\eta$  carried out predominantly error-prone and error-free TLS of dG- $N^2$ -IQ and dG-C8-IQ, respectively, whereas pol  $\kappa$  did exactly the opposite. It is also notable in Figure 9 that MF of dG- $N^2$ -IQ was only 28% higher than that of dG-C8-IQ at G<sub>1</sub>. In stark contrast, MF of dG-C8-IQ was 450% higher than that of dG- $N^2$ -IQ at G<sub>3</sub>. Since the decrease in TLS accompanied a decrease in MF from G<sub>1</sub> to G<sub>2</sub> to G<sub>3</sub>, one can normalize the MF by dividing it with TLS. The MF/TLS assessment provided values of 0.58, 0.52, and 0.36 for G<sub>1</sub>, G<sub>2</sub>, and G<sub>3</sub>, respectively, which may be considered a mutation per the TLS event. However, they still followed the same trend as MF alone. Taken together, these results underscore the importance of DNA sequence context on mutagenesis by carcinogen-DNA adducts. For both IQ-DNA adducts, MF was higher at the site where more facile TLS occurred.

High-resolution NMR studies revealed that dG- $N^2$ -IQ adopts a base-displaced intercalated conformation at both G<sub>1</sub> and G<sub>3</sub> but that in each case the modified guanine remains in the *anti* conformation about the glycosidic bond.<sup>48,49</sup> In contrast, the torsion angle is in *syn* conformation for the dG-C8-IQ adduct at each of the three guanine sites of *NarI* with the modified guanine displaced into the major groove, but base-displaced intercalated IQ is favored only at G<sub>3</sub>, in which it is flanked by guanines on both sides in the complementary strand,<sup>58,59</sup> and where it is most error-prone. When dG-C8-IQ was positioned at G<sub>1</sub> or G<sub>2</sub>, the IQ moiety was characterized as a minor groove bound.<sup>59</sup> DNA adducts localized in the solvent-exposed major groove, as in the case of dG-C8-IQ at G<sub>3</sub>, have been suggested to be better tolerated than adducts located in the minor groove as in the case of dG- $N^2$ -IQ that interact with the polymerase surface,<sup>60</sup> although this observation was made with a replicative pol. A more pertinent study is the one in which the major benzo[*a*]pyrene adduct at the dG- $N^2$  position in the minor groove is accommodated in *anti* conformation in the active site of pol  $\kappa$ .<sup>61</sup> The hydrophobic polycyclic ring system of the adduct is stabilized by the protein residues along the minor groove side to allow Watson–Crick base pairing with an incoming dCTP for accurate replication. The N-clasp domain of pol  $\kappa$  supports an open conformation, and similar interactions can be anticipated for the error-free bypass of dG- $N^2$ -IQ. When the adduct maintains its *syn* conformation, efficient destacking of the polycyclic aromatic moiety on top of the primer–template junction was shown to be a necessary step for efficient TLS by pol  $\eta$ .<sup>62</sup> The bypass, in such a case, is anticipated to be slow and error-prone. We also cannot rule out the possibility that of the various conformers of dG- $N^2$ -IQ at the three guanines of *NarI* sequence, the TLS pol can selectively bypass a minor conformer, which may escape detection in adduct structure investigations. For the erroneous TLS of dG- $N^2$ -IQ, the base-displaced intercalated *anti* orientation of the adduct may comprise a two or more



polymerase bypass model,<sup>36,37,63,64</sup> in which pol  $\eta$  incorporates dATP and pol  $\zeta$  carries out extension of the mispair, whereas Rev1 is involved in a noncatalytic role.

In conclusion, dG-N<sup>2</sup>-IQ is mutagenic in HEK293T cells inducing mainly G  $\rightarrow$  T transversions; no frameshift deletions were observed. In the *NarI* sequence, the lesion is most mutagenic when located at G<sub>1</sub>. Of the bypass pols, pol  $\kappa$  performs TLS of dG-N<sup>2</sup>-IQ in an error-free manner. In contrast, pol  $\eta$ , pol  $\zeta$ , and Rev1 cooperatively carry out the mutagenic TLS.

## ■ ASSOCIATED CONTENT

### ● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemrestox.6b00221.

*In vitro* experimental details, autoradiogram of the gel electrophoresis of the insertion of dCTP by human Rev1 opposite a control dG or dG-N<sup>2</sup>-IQ, and tables detailing mutation data (PDF)

## ■ AUTHOR INFORMATION

### Corresponding Author

\*University of Connecticut, Department of Chemistry, 55 N. Eagleville Rd, Storrs, CT 06269. Tel. 860-486-3965. Fax 860-486-2981. E-mail: ashis.basu@uconn.edu.

### Present Addresses

§A.B.: Hillman Cancer Center, University of Pittsburgh Cancer Institute, Pittsburgh, PA 15213.

||A.D.: Nitto Denko Avecia Inc., Cincinnati, OH 45215.

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### Notes

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## ■ ABBREVIATIONS

TLS, translesion synthesis; IQ, 2-amino-3-methylimidazo[4,5-f]quinoline; HEK, human embryonic kidney; MF, mutation frequency; pol, polymerase

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