Excision of 5-Halogenated Uracils by Human Thymine DNA Glycosylase

**ROBUST ACTIVITY FOR DNA CONTEXTS OTHER THAN CpG**

Michael T. Morgan, Matthew T. Bennett, and Alexander C. Drohat

From the Department of Biochemistry and Molecular Biology, Marlene and Stewart Greenebaum Cancer Center, University of Maryland School of Medicine, Baltimore, Maryland 21201

Thymine DNA glycosylase (TDG) excises thymine from G-T mispairs and removes a variety of damaged bases (X) with a preference for lesions in a CpG-X context. We recently reported that human TDG rapidly excises 5-halogenated uracils, exhibiting much greater activity for CpG-FU, CpG-CIU, and CpG-BrU than for CpG-T. Here we examine the effects of altering the CpG context on the excision activity for U, T, FU, CIU, and BrU. We show that the maximal activity ($k_{max}$) for G-X substrates depends significantly on the 5'-flanking pair. For example, $k_{max}$ decreases by 6-, 11-, and 82-fold for TpG-CIU, CpG-CIU, and ApG-CIU, respectively, as compared with CpG-FU. The other G-X substrates, the 5'-neighbor effects have a similar trend but vary in magnitude. The activity for G-FU, G-CIU, and G-BrU, with any 5'-flanking pair, meets and in most cases significantly exceeds the CpG-T activity. Strikingly, human TDG activity is reduced 10$^{2.3}$–10$^{4.3}$-fold for A-X relative to G-X pairs and reduced further for A-X pairs with a 5' pair other than C-G. The effect of altering the 5’ pair and/or the opposing base (G-X versus A-X) is greater for substrates that are larger (bromodeoxyuridine, dT) or have a more stable N-glycosidic bond (such as dT). The largest CpG context effects are observed for the excision of thymine. The potential role played by human TDG in the cytotoxic effects of CIU and BrU incorporation into DNA, which can occur under inflammatory conditions and in the cytotoxicity of FU, a widely used anticancer agent, are discussed.

The nucleobases in DNA are subject to continuous chemical modification, generating a broad range of mutagenic and cytotoxic lesions that can lead to cancer and other diseases (1, 2). To counteract this inevitable damage, the cellular machinery includes systems for DNA repair (3). Damage occurring to the nucleobases is the purview of base excision repair, a pathway that is initiated by a damage-specific DNA glycosylase. These enzymes find damaged or mismatched bases within the vast expanse of normal DNA and catalyze the cleavage of the base-sugar (N-glycosidic) bond, producing an abasic or apurinic/apyrimidinic (AP)$^2$ site in the DNA. The repair process is continued by follow-on base excision repair enzymes.

Human thymine DNA glycosylase (hTDG) was discovered as an enzyme that removes thymine from G-T and uracil from G-U mispairs in DNA (4, 5). In vertebrates, G-T mispairs arise from replication errors, which are handled by the mismatch repair pathway or from the deamination of 5-methylcytosine to T (6, 7). Because cytosine methylation occurs at CpG dinucleotides (8, 9), G-T mispairs caused by 5-methylcytosine deamination are found at CpG sites. It has been shown that hTDG is most active for G-T mispairs with a 5' C-G pair, suggesting that a predominant biological role of the enzyme is to initiate the repair of CpG-T lesions (10, 11). DNA methylation at CpG plays a fundamental role in many cellular processes, including transcriptional regulation and the silencing of repetitive genetic elements (8, 9). Suggesting a biological imperative to maintain the integrity of CpG sites, another human DNA glycosylase exhibits specificity for G-T mispairs at CpG sites; methyl binding domain IV (MBD4) (12–15).

In addition to its CpG-T activity, hTDG has been shown to remove a variety of damaged bases (5, 16–19), most of which are shown in Fig. 1. We recently identified several new hTDG substrates (20), including 5-chlorouracil (CIU), 5-iodouracil (IU), 5-flourouracine (FC), and 5-bromocytosine (BrC) (the activity is weak for IU, FC, and BrC and is probably not biologically relevant). The ability of hTDG to remove a broad range of damaged nucleobases is consistent in its relatively large and nonspecific active site (21, 22). Yet despite its substrate promiscuity, hTDG exhibits exceedingly weak activity for the excision of cytosine and 5-methylcytosine (11, 16, 20). We recently showed that for a broad range of C5-substituted uracil and cytosine bases, hTDG specificity depends on substrate reactivity (i.e., the stability of the scissile C-N bond) rather than the selective recognition of substrates in the active site (20). Moreover, we showed that specificity against the excision of cytosine from the huge excess of normal G-C pairs in DNA is largely explained by the very low reactivity of dC rather than the inability of hTDG to flip cytosine into its active site (20). Consistent
with this catalytic mechanism and the enhanced reactivity of 5-halogenated dU substrates, we found that hTDG rapidly excises FU, CIU, and BrU from CpG sites (20). Indeed, compared with CpG-T, the activity is 920-fold greater for CpG-FU, 550-fold greater for CpG-CIU, and 53-fold greater for CpG-BrU (20).

The robust activity observed for CpG-CIU and CpG-BrU suggests that hTDG may also have significant activity for removing CIU and BrU from DNA contexts other than CpG, raising the possibility that hTDG could play a role in the mutagenic and cytotoxic effects associated with CIU and BrU incorporation into DNA (23, 24). These lesions can arise in DNA when the 5-chloro-dUTP or 5-bromo-dUTP pools become elevated, which can be promoted by the activity of peroxidases during inflammation (25, 26). The very strong hTDG activity for CpG-FU substrates is also of interest because FU has been used for decades to treat many types of cancer (27). The mechanism of FU cytotoxicity is thought to involve multiple pathways, including a repetitive cycle of U and FU incorporation into DNA followed by the excision of these bases by a DNA glycosylase, increasing the burden abasic sites and leading to DNA strand breaks (27). Thus, hTDG could potentially be involved in the cytotoxicity of FU, as suggested by a report that inactivation of TDG in fission yeast and in mouse embryonic fibroblasts diminishes the sensitivity of these cells to FU treatment (28).

To further examine these possibilities, it is important to determine the activity of hTDG for removing FU, CIU, and BrU from DNA contexts other than CpG, because the incorporation of these bases into DNA or their presence in the template strand can be expected to give predominantly A-X pairs but also some G-X pairs and with no significant preference for a CpG sequence context. Although previous studies have examined the effect of altering the 5′-flanking pair on hTDG activity for G-T and G-C (10, 29), quantitative studies have not been reported for the many other hTDG substrates. Moreover, previous studies (and our findings here) indicate that the effect of the 5′-flanking pair depends strongly on the nature of the target base (29), so the results for G-T substrates do not necessarily predict the 5′-neighbor effects for other substrates. In addition, the effect of pairing the target base with adenine rather than guanine (i.e., A-X versus G-X) has not been rigorously examined for substrates other than U (10, 30), and the effect of altering the 5′-flanking pair for A-X substrates is completely unexplored.

Here, we use single turnover kinetics experiments to compare the activity of hTDG ($k_{\text{max}}$) for substrates that contain a G-X lesion with various 5′-flanking base pairs, i.e., CpG-X, TpG-X, GpG-X, and ApG-X, where X represents FU, CIU, BrU, U, or T. We also examine the effect of pairing the target base with adenine rather than guanine (i.e., CpA-X versus CpG-X). Finally, we examine the combined effect of pairing the target base with adenine and altering the 5′-flanking base pair using CpA-X, TpA-X, GpA-X, and ApA-X substrates. These studies provide the relative activity of hTDG for the excision of U, FU, CIU, and BrU from DNA contexts other than CpG, i.e., those in which they might be expected to arise in vivo. In addition, by systematically altering the CpG context for a series of target bases, our findings illuminate the catalytic role of the putative interactions that hTDG forms with the opposing guanine and with the 5′ C-G base pair.

### EXPERIMENTAL PROCEDURES

**DNA Synthesis and Purification**—Duplex DNA substrates were hybridized in 10 mM Tris, pH 8.0, 0.1 M NaCl, and 0.1 mM EDTA by rapid heating to 80 °C and slow cooling to room temperature. Single-strand DNA oligonucleotides were synthesized at the Biopolymer Genomics Core Facility, University of Maryland, Baltimore and at the Keck Foundation Biotechnology Resource Laboratory of Yale University. The 5-chlorodeoxyuridine phosphoramidite was obtained from ChemGenes Corp. (Wilmington, MA). Oligonucleotides were purified by anion exchange HPLC using a Zorbax Oligo column (Agilent Technologies), desalted by gel filtration using pre-packed Sephadex G25 columns (GE Healthcare), and stored at −20 °C. Oligonucleotide purity was verified by analytical anion-exchange HPLC under denaturing (pH 12) conditions using a DNA Pac PA200 column (Dionex Corp.), as described previously (20). Oligonucleotides were quantified by absorbance (260 nm) using the pairwise extinction coefficients, calculated as described (31).

**Expression and Purification of hTDG**—Escherichia coli BL21(DE3) cells (Stratagene) were transformed with a pET-28-based expression plasmid for human TDG, 410-amino acids in length) at 37 °C until absorbance (260 nm) using the pairwise extinction coefficients, calculated as described (31).

**Expression and Purification of hTDG**—Escherichia coli BL21(DE3) cells (Stratagene) were transformed with a pET-28-based expression plasmid for human TDG, 410-amino acids in length) at 37 °C until absorbance (260 nm) using the pairwise extinction coefficients, calculated as described (31).
Effect of CpG Context on 5-Halouracil Excision by hTDG

The lysate was cleared by centrifugation and incubated with 4 ml of nickel-nitrilotriacetic acid metal affinity resin (Qiagen) for 1 h at 4 °C. The lysate-resin mix was placed in a gravity-flow column, washed with 30 ml of lysis buffer containing 1 M NaCl and 20 mM imidazole followed by 30 ml of lysis buffer containing 20 mM imidazole, and hTDG was eluted with lysis buffer containing 150 mM imidazole. hTDG was purified further using an SP-Sepharose HP column (GE Healthcare) with buffers IE-A (25 mM Tris, pH 7.5, 75 mM NaCl, 1 mM dithiothreitol, 0.2 mM EDTA, 1% glycerol) and IE-B (IE-A with 1 mM NaCl) and a gradient of 5–20% IE-B over 60 min at 2.5 ml/min. hTDG was further purified using a Q-Sepharose HP column (GE Healthcare) with the same IE-A and IE-B buffers and a gradient of 0–100% IE-B over 60 min at 2.5 ml/min. The purity of hTDG was >99% as judged by SDS-PAGE stained with Coomassie. Purified hTDG was dialyzed overnight versus storage buffer (20 mM HEPES 7.5, 0.1 mM NaCl, 1 mM dithiothreitol, 0.5 mM EDTA, 1% glycerol), concentrated to about 0.1 mM, flash-frozen in small aliquots, and stored at −80 °C. The concentration of hTDG was determined by absorbance using ε280 = 31.5 mM−1 cm−1 (33).

Single Turnover Kinetics—Because hTDG is strongly inhibited by its abasic DNA product (10), the rate constant obtained from steady-state kinetics experiments (kcat) is dominated by product release and is not useful for comparing hTDG activity for various substrates. Here, we use single turnover kinetics under saturating enzyme conditions to obtain rate constants (kmax) that are not impacted by product release or the association of enzyme and substrate and thereby reflect the maximal enzymatic activity for a given substrate. To ensure that the observed rate constants represent the maximal value (i.e. kabs = kmax), single turnover experiments were collected using a large excess of enzyme over substrate and with an enzyme concentration that is more than 100-fold higher the Kapp = 41 mM reported as the apparent binding affinity of hTDG for DNA containing a CpG:T mispair (29). To confirm saturating enzyme conditions, experiments were in some cases conducted with two or more hTDG concentrations, typically 5 and 10 μM, providing rate constants that were equivalent within experimental uncertainty. Substrate DNA concentrations were 500 nM unless noted otherwise. Single turnover reactions were performed either manually or using a rapid chemical quenched-flow instrument (RQF-3, Kintek Corp.). The reactions were conducted at 22 °C in HEMN1 buffer (20 mM HEPES, pH 7.50, 0.2 mM EDTA, 2.5 mM MgCl2, 0.1 mM NaCl, 1 mM dithiothreitol, 0.5 mM EDTA, 1% glycerol), and heated for 15 min at 85 °C to induce cleavage of the DNA backbone at AP sites. The extent of product formation was analyzed by HPLC, as described below. Rate constants were determined by fitting the single turnover data to a single exponential equation using nonlinear regression with Grafit 5 (34). In most cases, the reactions proceeded to full completion, except those that are very slow (i.e. kmax < ~1 × 10−4 min−1).

**HPLC Assay for Monitoring hTDG Activity**—We recently developed a HPLC assay for monitoring hTDG activity (20). Samples taken during a kinetics experiment contain a mixture of substrate and products that is comprised of four oligonucleotides; that is, the full-length target strand and its complement and two shorter strands resulting from alkaline cleavage of the nascent abasic strand. These strands are resolved by anion exchange HPLC using denaturing (pH 12.0) conditions with a DNA Pac PA200 column (Dionex Corp.). The alkali conditions serve to suppress hybridization of ssDNA during chromatography and have the added benefit of resolving strands that are of the same length but differ in the number of thymine and guanine bases, which are negatively charged at pH 12. The elution buffer is 0.02 mM sodium phosphate pH 12.0 containing either 0.03 mM NaClO4 (buffer A) or 0.50 mM NaClO4 (buffer B). The oligonucleotides are detected by absorbance (260 nm), and the fraction product (I) is determined from the integrated peak areas for the target strand (A0) and product strands (A1 and A2) using the equation F = (A1 + A2)/(A0 + A1 + A2). The determination of fraction product using this assay is reproducible to within 1%, as determined from multiple analyses of identical samples.

**RESULTS**

In a recent study we determined the activity of hTDG (kmax) for a series of 5-substituted uracil and cytosine substrates in which the target base was placed in a CpG context (20). We found that kmax is much higher for CpG:INT, CpG:GUC and CpG:BrU than for CpG:T, suggesting that hTDG may have significant activity for FU, CIU, and BrU in DNA contexts other than CpG. Here, we examine the effect of altering the CpG site context on hTDG activity for the excision of T, U, FU, CIU, and BrU for the YpG substrates the 5′-flanking pair is varied for G-X substrates. For example, YpG-XT indicates that the G-X target pair has a 5′-flanking T-A pair. For the CPAGX substrates the target base is paired with A rather than G, whereas the 5′-CG pair is retained. For the Ypα-X substrates, the target base is paired with A, and the 5′-flanking base pair is varied.

**CpG-X: target in normal CpG site**
- substrate: 5′GCTCTGTACCTGAGCAGTG
- complements: 3′CGAGACATGTACTCCGTAC

**YpG-X: vary the 5′ pair for G-X**
- complement: 5′GCTCTGTAYTGAGCAGTG
- substrate: 3′CGAGACATZXACTCCGTAC

**CpA-X: target base paired with A**
- complement: 5′GCTCTGTACATGAGCAGTG
- substrate: 3′CGAGACATZXACTCCGTAC

**YpA-X: vary the 5′ pair for A-X**
- complement: 5′GCTCTGTAYATGAGCAGTG
- substrate: 3′CGAGACATZXACTCCGTAC
sic DNA (10, 14, 35–37). Indeed, previous studies show that under limiting enzyme conditions, the turnover of hTDG is exceedingly slow after it converts one molar equivalent of G·T (or G·U) substrate to G·AP product (10, 38, 39). Thus, the rate constant obtained from steady-state kinetics, \( k_{\text{cat}} \), is dominated by product release and cannot provide a meaningful comparison of activity for different substrates (Fig. 3). In contrast, single turnover kinetics conducted under saturating enzyme conditions provide a rate constant (\( k_{\text{max}} \)) that is not impacted by product release or the association of enzyme and substrate and, therefore, reflects the maximal activity for a given substrate (Fig. 3). For the hTDG reaction, \( k_{\text{max}} \) reflects the rate constant for the chemical step (\( k_{\text{chem}} \)) and is also influenced by the equilibrium constant for base flipping (\( K_{\text{flip}} \)). In the base-flipping step, the target nucleotide flips out of the DNA duplex and into the active site, a process that likely involves a conformational change in hTDG, as observed for uracil DNA glycosylase (40). Thus, differences in \( k_{\text{max}} \) that result from alterations to the CpG context reflect a change in \( k_{\text{chem}} \) and/or \( K_{\text{flip}} \).

**Effect of the 5′ Base Pair on 5-Halouracil Excision by hTDG**—We determined the effect of varying the 5′ neighboring base pair on hTDG activity (\( k_{\text{max}} \)) for G·FU, G·CIU, G·BrU, G·U, and G·T using the YpG·X series of substrates (Fig. 2). The results are given in Table 1 and Fig. 4. Previous studies showed that hTDG activity for G·T sub-

![Figure 3: Minimal kinetic mechanism for hTDG. Shown is a minimal kinetic mechanism for hTDG, including the steps that comprise \( k_{\text{cat}} \), which is obtained from steady-state kinetics, and \( k_{\text{max}} \), as obtained from single turnover kinetics with a saturating enzyme concentration (used here). Association of hTDG (E) and DNA substrate (D) forms the initial collision complex, E·D, and base-flipping (\( K_{\text{flip}} \)) gives the reactive enzyme-substrate complex, E·D (where D is base-flipped DNA). Base flipping likely involves a conformational change for hTDG, which is not explicitly shown. The chemical step (\( k_{\text{chem}} \)) involves cleavage of the base-sugar (N-glycosidic bond) and the addition of the water nucleophile, producing the ternary product complex, E·B·apD (B is the nucleobase, apB is abasic DNA). Release of the excised base likely precedes the dissociation of abasic DNA, which is known to be very slow. As shown, \( k_{\text{max}} \) is influenced by \( k_{\text{flip}} \) and \( k_{\text{chem}} \), where \( k_{\text{max}} = k_{\text{flip}} (k_{\text{flip}} + k_{\text{chem}}) \).](image)

**TABLE 1**

| Substrate | \( k_{\text{max}} \) \( \text{Min} \^{-1} \) | -Fold change relative to CpG·X | -Fold change relative to CpG·T | -Fold change relative to CpA·X | -Fold change relative to YpG·X |
|-----------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| G·X       |                                 |                                 |                                 |                                 |                                 |
| CpG·T     | 0.22 ± 0.04                     | 1                               | 1                               |                                 |                                 |
| TpG·T     | 0.0060 ± 0.0001                 | 0.027                           | 0.027                           |                                 |                                 |
| CpG·C     | 0.0023 ± 0.0002                 | 0.010                           | 0.010                           |                                 |                                 |
| ApG·T     | 0.000038 ± 0.00005              | 0.0017                          | 0.0017                          |                                 |                                 |
| CpG·U     | 2.6 ± 0.3                       | 1                               | 12                              |                                 |                                 |
| TpG·U     | 0.79 ± 0.04                     | 0.303                           | 3.6                             |                                 |                                 |
| CpG·B     | 0.88 ± 0.11                     | 0.340                           | 4.0                             |                                 |                                 |
| ApG·U     | 0.117 ± 0.003                   | 0.045                           | 0.5                             |                                 |                                 |
| CpG·FU    | 202 ± 16                        | 1                               | 918                             |                                 |                                 |
| TpG·FU    | 113 ± 1                        | 0.558                           | 513                             |                                 |                                 |
| CpG·FU    | 125 ± 11                        | 0.618                           | 568                             |                                 |                                 |
| ApG·FU    | 18 ± 1                         | 0.089                           | 75                              |                                 |                                 |
| CpG·CIU   | 120 ± 6                        | 1                               | 546                             |                                 |                                 |
| TpG·CIU   | 20.9 ± 0.5                     | 0.174                           | 95                              |                                 |                                 |
| CpG·CIU   | 11.1 ± 0.3                     | 0.093                           | 51                              |                                 |                                 |
| ApG·CIU   | 1.46 ± 0.15                    | 0.012                           | 6.7                             |                                 |                                 |
| CpG·BrU   | 11.6 ± 1.0                     | 1                               | 53                              |                                 |                                 |
| TpG·BrU   | 1.2 ± 0.1                     | 0.106                           | 5.6                             |                                 |                                 |
| CpG·BrU   | 0.44 ± 0.06                    | 0.038                           | 2.0                             |                                 |                                 |
| ApG·BrU   | 0.15 ± 0.02                    | 0.013                           | 0.7                             |                                 |                                 |

\( \text{ND}, \text{Not determined.} \)

\( ^{a} \text{The rate constants (} k_{\text{max}} \text{) reflect the maximal enzymatic activity of hTDG for a given substrate, as determined using single turnover kinetics experiments with saturating enzyme conditions.} \)

\( ^{b} \text{-Fold change relative to YpG·X gives the effect of pairing the target base (X) with adenine rather than guanine for a given 5′ base pair (Y), i.e. the rate of TpA·U relative to TpG·U.} \)
Effect of CpG Context on 5-Halouracil Excision by hTDG

**FIGURE 4. Effect of the 5′-flanking base pair on hTDG activity for G-X substrates.** A, bar graph shows the maximal activity of hTDG (log $k_{max}$) for the various TpG-X substrates. For comparison, the activity for CpgT ($k_{max} = 0.22$ min$^{-1}$) is indicated by the dotted line. B, representative data from single turnover kinetics experiments for FU excision from CpgFU (○), TpgFU (■), GpgFU (□), and ApgFU (△) substrates. The inset shows the complete time course for ApgFU (△). C, representative data from single turnover kinetics for CIU excision from CpgCIU (○), TpgCIU (■), GpgCIU (□), and ApgCIU (△) substrates. The inset shows the complete data for ApgCIU (△).

**FIGURE 5. hTDG activity is greatly reduced for A-X relative to G-X substrates.** The hTDG activity (log $k_{max}$) for CpgX versus CpA-X substrates is shown. The change in log $k_{max}$ is given.

The hTDG activity depends strongly on the 5′-flanking pair, with relative activity of CpgG>T >> TpgG>T > GpgG>T > ApgG>T (10, 11). We find a similar trend here; compared with CpgG-T, $k_{max}$ is reduced by 37-, 96-, and 582-fold for TpgG-T, GpgG-T, and ApgG-T, respectively. The influence of the 5′ neighbor is much smaller for G-U activity; compared with CpgG-U, $k_{max}$ is decreased by 3.3-, 2.9-, and 22-fold for TpgG-U, GpgG-U, and ApgG-U, respectively. The 5′-neighbor effect is also small for F-U activity; compared with CpgF-U, $k_{max}$ decreases by merely 1.8-, 1.6-, and 11-fold for TpgF-U, GpgF-U, and ApgF-U, respectively (Fig. 4B). The 5′-neighbor effects are much larger for G-FIU activity; compared with CpgG-FIU, $k_{max}$ is decreased by 6-, 11-, and 82-fold for TpgG-FIU, GpgG-FIU, and ApgG-FIU, respectively (Fig. 4C). The results are similar for G-Bru; compared with CpgG-Bru, $k_{max}$ is decreased by 9-, 26-, and 75-fold for TpgG-Bru, GpgG-Bru, and ApgG-Bru, respectively.

**Activity Is Greatly Diminished for A-X Relative to G-X Substrates**—It has been reported that hTDG does not remove thymine from A-T pairs (10, 11, 41), in keeping with the biological imperative to minimize the activity against undamaged DNA. We, therefore, wondered to what extent the robust hTDG activity observed for G-FU, G-FIU, and G-Bru is diminished when the 5′-halouracil bases are paired with adenine rather than guanine. This is relevant because 5-halogenated dU is incorporated opposite template dA and vice versa during replication (24). We examined the effect of pairing the target base with adenine rather than guanine while preserving the 5′ C-G pair using the CpaA-X substrates (Fig. 2). The data are listed in Table 1 and displayed in Fig. 5. Under the experimental conditions used here, i.e. high concentrations of substrate (500 nM) and enzyme (5 μM), we were able to measure the exceedingly weak activity for thymine excision from the CpaA-T substrate, $k_{max} = 1.3 \times 10^{-5}$ min$^{-1}$. This is a striking 17,600-fold lower than the activity for CpgG-T. In contrast, a much smaller 795-fold decrease is observed for CpaA-U compared with CpgG-U, consistent with a previously reported 600-fold decrease (30). Similarly, a small 187-fold decrease is observed for CpaA-FU relative to CpgG-FU. For the excision of CIU, which is similar to T in terms of its steric and electrostatic properties (20), we find a much larger 5460-fold decrease for CpaA-CIU versus CpgG-CIU. A significantly larger 14,870-fold decrease is
Effect of CpG Context on 5-Halouracil Excision by hTDG

DISCUSSION

Role of the 5'-C-G Pair on G-X Activity—The effect of altering the 5'-C-G pair on G-X activity was examined using the YpG-X series of substrates (Fig. 2), and the results are given in Table 1 and Fig. 4A. Our findings indicate that the magnitude of the 5'-neighbor effect depends on the size of the nucleobase and on substrate reactivity, i.e., the stability of the C-N bond that is cleaved by hTDG. For the uracil analogues examined here, size depends on the C5 substituent, with nucleobase volume varying as U < FU < CIU < BrU (20). The substrate reactivity (N-glycosidic bond stability) also depends on the C5 substituent. Thus, for the hTDG-catalyzed and non-catalyzed reactions, the rate depends on the leaving ability of the departing nucleobase (20, 42–44). The electron withdrawing halogens enhance the leaving ability of uracil, whereas the electron donating methyl group suppresses it. Accordingly, substrate reactivity varies as: ClU ≥ BrUrd ≥ FdU > dU > dT (20). As we consider the effects of altering the CpG context, we note that differences in $k_{\text{max}}$ reflect a change in the equilibria for the base flipping step ($k_{\text{flip}}$) and/or the rate of the chemical step ($k_{\text{chem}}$), as shown in Fig. 3.

The dependence of the 5'-neighbor effect on substrate reactivity is illustrated by comparing the effects for G-CIU, ranging from 6- to 82-fold, with the effects for G-T, which range from 37- to 582-fold. As we have noted previously, the size and electrostatic properties of T and CIU are quite similar (20), suggesting that T and CIU are not substantially discriminated by the relatively large and nonspecific hTDG active site (21, 22). In addition, melting studies showed that G-T and G-CIU base pairs have essentially the same stability in DNA, indicating that CIU and T have a similar propensity for flipping spontaneously out of the duplex (20). This suggests that the steeper dependence of $k_{\text{max}}$ on the 5'-flanking pair for G-T relative to G-CIU is not due to substantial effects on the base-flipping step. The most significant difference is that ClU is much more reactive than dT, which suggests that altering the 5'-C-G pair elicits a greater effect on $k_{\text{chem}}$ for G-T than for G-CIU pairs. Thus, interactions with the 5' C-G pair may induce a conformational change in hTDG and/or the DNA substrate that stabilizes the transition state, and this effect appears to be greater for less reactive substrates.

The dependence of the 5'-neighbor effect on substrate size is illustrated by comparing the effects for G-FU, 2–11-fold, with G-BrU, 9–75-fold. The reactivity of FdU and BrdUrd is about the same as is the stability of G-FU and G-BrU base pairs in DNA; however, BrU is substantially larger than FU (20). Although the hTDG active site is accommodating, we found previously that $k_{\text{max}}$ decreases for substrates with large C5 substituents, including BrU, 5-iodouracil, and 5-bromocytosine (20). Thus, our results suggest that interactions with the 5'-C-G pair serve to increase $k_{\text{flip}}$, stabilize the transition state of the reaction and that the effect is greater for substrates with a larger C5-group (BrdUrd and dT). A previous study found that the apparent $K_p$ is nearly the same for CpG-T and TpG-T substrates, suggesting that recognition of the 5'-C-G pair contributes largely to stabilizing the transition state (increasing $k_{\text{chem}}$) rather than promoting base flipping (29). However, additional

![Figure 6. Effects of altering the 5'-flanking pair on hTDG activity for A-X pairs. Shown is the hTDG activity (log $k_{\text{max}}$) for the YpA-X substrates, which contain an A-X pair with one of four possible 5'-flanking pairs. For comparison, the activity for CpG-T ($k_{\text{max}} = 0.22 \text{ min}^{-1}$) is indicated by the dotted line.](image-url)
studies are required to establish which steps of the hTDG reaction are affected by recognition of the 5’ C-G pair.

**Effect of Pairing the Substrate Base with A Rather than G**—Because hTDG excises a normal base, thymine, from G-T mismatches, it has an effective mechanism for avoiding the excision of T from the huge excess of A-T pairs in DNA (10, 11). The specificity against A-T may involve H-bond interactions that select for guanine, as observed in a crystal structure of the related mismatch uracil DNA glycosylase from *E. coli* (eMUG) (45). Here, we have quantitatively established the specificity of hTDG for excising bases from G-X versus A-X pairs, and we find that it is strikingly large. The difference in $k_{\text{max}}$ ranges from 187-fold for Cpa-A-FU versus Cpa-G-FU to 17,600-fold for Cpa-A versus Cpa-G (Table 1, Fig. 5). Our findings indicate that these large differences depend on the size and reactivity (C-N bond stability) of the nucleotide substrate. The much larger effect for BrdUrd (10^{1.2}-fold) relative to FdU (10^{2.3}-fold) is likely due to the larger size of BrdUrd because these substrates have similar reactivity (20). The larger effect for dU (10^{2.9}-fold) compared with FdU is probably attributable to the significantly greater reactivity of FdU, since dU and FdU have relatively similar steric and electrostatic properties (20). Thus, our results suggest that the putative interactions formed with the mismatched guanine serve to promote base-flipping (increase $k_{\text{flip}}$) and/or stabilize the transition state (increase $k_{\text{chem}}$) and that disrupting these interactions has a greater effect for larger and less reactive substrates (i.e. dT). Additional mechanistic and/or structural studies are needed to determine how hTDG selects for G-X over A-X pairs and which step(s) of the reaction is involved.

It also of interest to consider the difference in activity for A-X versus G-X for substrates in which the 5’-flanking pair is not C-G. These effects are listed in Table 1 under “Fold change relative to YpG-X.” For example, considering U excision, the decrease in $k_{\text{max}}$ is 10^{3.0}-fold for TpA-U versus TpG-U, 10^{4.2}-fold for GpA-U versus GpG-U, and 10^{5.6}-fold for ApA-U versus ApG-U as compared with 10^{2.9}-fold for Cpa-U versus Cpa-G. The effects for the FU substrates have the same trend but are smaller in magnitude, whereas the effects for the CIU and BrU substrates have a similar trend and are larger in magnitude. Overall, the results indicate that the difference in activity for A-X versus G-X substrates is similar when the 5’-flanking pair is C-G or T-A, becomes larger for a 5’ A-T pair, and is maximal for a 5’ G-C pair.

**Combined Effect of Pairing the Target Base with Adenine and Altering the 5’ C-G Pair**—It is illuminating to consider the effect on $k_{\text{max}}$ of replacing the opposing guanine with adenine and altering the 5’-C-G pair. The combined effects are very large, as shown in the lower region of Table 1 under the heading “Fold change relative to CpG-X.” For example, compared with the activity for Cpa-G, $k_{\text{max}}$ is reduced by 10^{3.5}-, 10^{4.7}-, and 10^{4.7}-fold for TpA-U, GpA-U, and ApA-U, respectively. Similar reductions in activity in 10^{2.9} to 10^{3.5}-fold are seen for the FU substrates. The effects are even more striking for the larger bases, ranging from 10^{4.4} to 10^{6.4}-fold for ClU and 10^{4.8} to 10^{6.2}-fold for BrU. The huge effects observed for CIU and BrU suggest that the specificity of hTDG for CpG-T lesions over normal A-T pairs ranges in magnitude from 10^{4.3} to 10^{6.4}-fold or more, corresponding to 6–8.7 kcal/mol.

**Implications for CpG-T Specificity**—Taken together our findings indicate that disrupting the interactions that hTDG forms with the 5’ C-G pair and the mismatched guanine have a greater effect for substrates with a large C5-substituent and/or a more stable N-glycosidic bond. Thus, the relatively large size and low reactivity of dT likely explains the large (37–625-fold) 5’-neighbor effect on $k_{\text{max}}$ and the huge (10^{6.2}-fold) difference in activity for Cpa-A versus Cpa-G, as compared with a 32-fold difference for G-U activity and the much smaller difference between Cpa-U and Cpa-G (10^{3.9}-fold) is likely explained by the substantially smaller size and enhanced reactivity of dU relative to dT. These differences in activity would seem to be consistent with the requirement of the cell to restrict thymine excision to thymines that arise by 5-methylcytosine deamination at CpG sites, whereas uracil can be removed wherever it arises in DNA.

**Biomedical Relevance of FU Activity**—Our finding of very strong hTDG activity for G-FU lesions with any 5’ base pair, some 75–920-fold greater than CpG-T activity, may be relevant to the cytotoxicity of FU, which has been used for decades to treat cancer (27). The anticancer effect of FU is thought to involve multiple pathways, including the incorporation of U and FU into DNA followed by their excision by a DNA glycosylase, leading to a cycle of incorporation and excision, an increased level of abasic sites, DNA fragmentation, and cell death (24, 27). Consistent with a potential role for TDG in this process, it was reported that TDG inactivation diminishes the sensitivity of fission yeast and mouse embryo fibroblasts to FU treatment and leads to a decrease in FU-induced DNA strand breaks (28). This likely reflects TDG activity against G-FU lesions because its A-FU activity is relatively weak. Other DNA glycosylases that could potentially elicit a similar effect by removing misincorporated U and/or FU include uracil DNA glycosylase (UNG2), SMUG1, and MBD4. The removal of U probably involves UNG2 and SMUG1 (46) because TDG and MBD4 are much less efficient against U (Table 1) (14). Although UNG2 has the highest activity for U removal, its activity is much lower for FU (47–49), and UNG2 does not remove FU from DNA in mouse embryo fibroblasts (49). Although hMBD4 has significant activity for CpG-FU lesions, the effect of the 5’ base pair on G-FU activity is unknown for this CpG specific enzyme, and it is inactive against A-FU pairs (50). Recent studies report that MBD4 inactivation decreases the
sensitivity of mice to FU treatment, although this may reflect a loss of an MBD4-mediated apoptotic response to DNA damage rather than FU excision (51).

The toxicity of FU may also depend on its presence in DNA due to mutagenesis or perturbations of protein–DNA interactions as was suggested by a report that FU excision by SMUG1 protects mouse embryo fibroblasts against FU-induced toxicity (49). The potential for mutagenesis is significant; FU incorporation yields mostly A-FU pairs but also some G-FU pairs, which can cause G-C → A-T mutations (52, 53). Thus, repair of G-FU lesions for which G is in the parental strand would be protective. However, because G can be incorporated opposite template FU, replication of A-FU pairs can give G-FU lesions, leading to A-T → G-C mutations (52, 53). It is important to note that base excision repair processing of these G-FU lesions would facilitate the A-T → G-C transition rather than protect against it. Perhaps the protective role observed for SMUG1 (49) reflects the repair of initial A-FU lesions (i.e. FU in the daughter strand). The reported effect of hTDG in enhancing the sensitivity of mouse embryo fibroblasts to FU treatment may reflect hTDG activity against G-FU lesions in which FU is in the parental strand or the production of abasic sites as discussed above.

**Biological Relevance of BrU and CIU Activity**—We find that hTDG exhibits robust activity for G-CIU and G-BrU pairs, which meets and in most cases exceeds the activity for CpdG-T (Fig. 4). Our findings may have important biological implications because it has been shown that CIU and BrU arise in DNA due to oxidative processes associated with inflammation (25, 26), leading to mutagenic, genotoxic, and cytotoxic effects (23, 24). As an element of host defense, peroxidases produce hypochlorous and hypobromous acid, which promote the halogenation of pyrimidines (at C5), leading to 5-chloro-dUTP and 5-bromo-dUTP (25, 26, 54–58). These dTTP analogues are incorporated into DNA, giving predominantly A-CIU and A-BrU pairs and some G-CIU and G-BrU lesions (53, 59), which can cause G-C → A-T mutations. The initial A-CIU and A-BrU pairs are also mutagenic, because incorporation of G opposite template CIU or BrU gives G-CIU or G-BrU and eventually A-T → G-C transitions (53, 59, 60). Until recently, it did not appear that any human enzymes could remove CIU or BrU from DNA. It had been shown that UNG2 and SMUG1 do not remove CIU or BrU likely due to steric hindrance in the active site (25, 61–63). Our findings raise the possibility that hTDG is active against G-CIU or G-BrU lesions that arise in vivo, and a recent study suggests that hMBD4 may also be active against these lesions (50). However, A-CIU and A-BrU lesions are poor substrates for hTDG (Table 1) and hMBD4 (50), and these lesions may persist in DNA. The effect of excising CIU or BrU may depend on whether the halogenated base is misincorporated or resides in the template strand. The excision of CIU or BrU that was misincorporated opposite template G could protect against G-C → A-T mutations. On the other hand, repair of G-CIU and G-BrU lesions that have arisen from replication of A-CIU or A-BrU (i.e. template CIU or BrU) will facilitate an A-T → G-C transition rather than protect against it. In addition to these potential effects on CIU- and BrU-induced mutagenesis, hTDG could potentially contribute to a repetitive cycle of CIU and BrU misincorporation and excision, leading to abasic sites and DNA strand breaks, similar to the potential role played by hTDG in FU toxicity, as discussed above.

It has been shown that sister-chromatid exchange increases with the amount of CldU and BrdUrd present in replicated DNA (64, 65). Although the mechanism of CIU- and BrdU-induced sister-chromatid exchange has not been established, evidence suggests that one mechanism involves the formation of single-strand breaks that originate from abasic sites (23, 24, 66). The generally accepted model is that these abasic sites arise from dehalogenation of CIU or BrU followed by excision of the resulting uracil by UNG2 (66). Our findings suggest an alternative mechanism, that the abasic sites originate from the direct excision of CIU and BrU by hTDG (and potentially MBD4). It has also been observed that CldU induces 3–5 times more sister-chromatid exchange than BrdUrd when these dT analogs are incorporated at equivalent level in DNA (24, 64). Our findings offer a potential explanation: the ~10-fold higher activity of hTDG for G-CIU over G-BrU could generate more abasic sites given a similar level of CIU and BrU incorporation into DNA. Thus, our findings and recent observations for MBD4 raise the possibility that these enzymes might contribute to the mechanism of CldU- and BrdUrd-induced sister-chromatid exchange.

**REFERENCES**

1. Lindahl, T. (1993) *Nature* **362**, 709–715
2. Loeb, L. A., and Christians, F. C. (1996) *Mutat. Res.* **350**, 279–286
3. Lindahl, T., and Wood, R. D. (1999) *Science* **286**, 1897–1905
4. Wibauer, K., and Jiricny, J. (1989) *Nature* **339**, 234–236
5. Neddermann, P., and Jiricny, J. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 1642–1646
6. Coulondre, C., Miller, J. H., Farabaugh, P. J., and Gilbert, W. (1978) *Nature* **274**, 775–780
7. Rideout, W. M., III, Coetzee, G. A., Olumi, A. F., and Jones, P. A. (1990) *Science* **249**, 1288–1290
8. Jones, P. A., and Takai, D. (2001) *Science* **293**, 1068–1070
9. Feinberg, A. P., and Tycko, B. (2004) *Nat. Rev. Cancer* **4**, 143–153
10. Waters, T. R., and Swann, P. F. (1998) *J. Biol. Chem.* **273**, 20007–20014
11. Sibghat, U., Gallinari, P., Xu, Y. Z., Goodman, M. F., Bloom, L. B., Jiricny, J., and Day, R. S., III (1996) *Biochemistry* **35**, 12926–12932
12. Hendrich, B., Hardeland, U., Ng, H. H., Jiricny, J., and Bird, A. (1999) *Nature* **401**, 301–304
13. Bellacosa, A., Cicchillitti, L., Schepis, F., Riccio, A., Yeung, A. T., Matsumoto, Y., Golemis, E. A., Genuardi, M., and Neri, G. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 3969–3974
14. Petronzelli, F., Riccio, A., Markham, G. D., Seeholzer, S. H., Stoerker, J., Genuardi, M., Yeung, A. T., Matsumoto, Y., and Bellacosa, A. (2000) *J. Biol. Chem.* **275**, 32422–32429
15. Millar, C. B., Guy, J., Sansom, O. J., Selfridge, J., MacDougall, E., Hendrich, B., Keightley, P. D., Bishop, S. M., Clarke, A. R., and Bird, A. (2002) *Science* **297**, 403–405
16. Hardeland, U., Bentele, M., Jiricny, J., and Schar, P. (2003) *Nucleic Acids Res.* **31**, 2261–2271
17. Liu, P., Burdzy, A., and Sowers, L. C. (2003) *DNA Repair (Anast)* **2**, 199–210
18. Saparbaev, M., and Laval, J. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 8508–8513
19. Yoon, J. H., Iwai, S., O’Connor, T. R., and Pleifer, G. P. (2003) *Nucleic Acids Res.* **31**, 5399–5404
20. Bennett, M. T., Rodgers, M. T., Hebert, A. S., Ruslander, L. E., Eisele, L., and Drohat, A. C. (2006) *J. Am. Chem. Soc.* **128**, 12510–12519
21. Barrett, T. E., Scharer, O. D., Savva, R., Brown, T., Jiricny, J., Verdone, G. L., and Pearl, L. H. (1999) *EMBO J.* **18**, 6599–6609
22. Baba, D., Maita, N., Jee, J.-G., Uchimura, Y., Saitoh, H., Sugasawa, K.,
Effect of CpG Context on 5-Halouracil Excision by hTDG

Hanaoka, F., Tochio, H., Hiroaki, H., and Shirakawa, M. (2005) Nature 435, 979–982
23. Morris, S. M. (1991) Mutat. Res. 258, 161–188
24. Morris, S. M. (1993) Mutat. Res. 297, 39–51
25. Jiang, Q., Blount, B. C., and Ames, B. N. (2003) J. Biol. Chem. 278, 32834–32840
26. Henderson, J. P., Byun, J., Takeshita, J., and Heinecke, J. W. (2003) J. Biol. Chem. 278, 23522–23528
27. Longley, D. B., Harkin, D. P., and Johnston, P. G. (2003) J. Biol. Chem. 278, 29587–29592
28. Cortazar, D., Kunz, C., Saito, Y., Steinacher, R., and Schar, P. (2007) J. Biol. Chem. 282, 15935–15943
29. Abu, M., and Waters, T. R. (2003) J. Biol. Chem. 278, 20526–20532
30. Wibley, J. E., Waters, T. R., Haushalter, K., Verdine, G. L., and Pearl, L. H. (1998) Mol. Pharmacol. 53, 389–403
31. Fasman, G. (1975) CRC Handbook of Biochemistry and Molecular Biology, 3rd Ed., CRC Press, Boca Raton, FL
32. Hardeland, U., Steinacher, R., Jiricny, J., and Schar, P. (2002) Biochemistry 41, 616–623
33. Gill, S. C., and von Hippel, P. H. (1989) Anal. Biochem. 182, 319–326
34. Leatherbarrow, R. J. (1998) Grafit 5, Erithacus Software Ltd., Staines, UK
35. O’Neill, R. J., Vorob’eva, O. V., Shahbakhti, H., Zmuda, E., Bhagwat, A. S., and Baldwin, G. S. (2003) J. Biol. Chem. 278, 20526–20532
36. O’Neill, J. P., Heartlein, M. W., and Preston, R. J. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 5741–5745
37. Steinacher, R., and Schar, P. (2005) Curr. Biol. 15, 616–623
38. Steinacher, R., and Jiricny, J. (1993) J. Biol. Chem. 268, 21218–21224
39. Shapiro, R., and Danzig, M. (1972) Biochemistry 11, 23–29
40. Jiang, Y. L., and Stivers, J. T. (2002) Biochemistry 41, 11236–11247
41. Neddermann, P., and Jiricny, J. (1993) J. Biol. Chem. 268, 21218–21224
42. Shapiro, R., and Kang, S. (1969) Biochemistry 8, 1806–1810
43. Vanschepdael, A., Macken, E., Busson, R., Janssen, G., Herdewijn, P., Roets, E., and Hoogmartens, J. (1993) J. Chromatogr. A 657, 208–212
44. Barrett, T. E., Savva, R., Panayotou, G., Barlow, T., Brown, T., Jiricny, J., and Pearl, L. H. (1998) Cell 92, 117–129
45. Krokan, H. E., Drablos, F., and Slupphaug, G. (2002) Oncogene 21, 8935–8948
46. Mauro, D., De Riel, I., Tallarida, R., and Sirover, M. (1993) Mol. Pharmacol. 43, 854–857
47. Kavli, B., Sundheim, O., Akbari, M., Otterlei, M., Nilsen, H., Skorpen, F., Aas, P. A., Hagen, L., Krokan, H. E., and Slupphaug, G. (2002) J. Biol. Chem. 277, 39926–39936
48. An, Q., Robins, P., Lindahl, T., and Barnes, D. E. (2007) Cancer Res. 67, 940–945
49. Turner, D. P., Cortellino, S., Schupp, J. E., Caretti, E., Loh, T., Kinsella, T. J., and Bellacosa, A. (2006) Cancer Res. 66, 7686–7693
50. Sansom, O. J., Zabkiewicz, J., Bishop, S. M., Guy, J., Bird, A., and Clarke, A. R. (2003) Oncogene 22, 7130–7136
51. Sowers, L., Eritja, R., Kaplan, B., Goodman, M., and Fazakerley, G. (1988) J. Biol. Chem. 263, 14794–14801
52. Yu, H., Eritja, R., Bloom, L., and Goodman, M. (1993) J. Biol. Chem. 268, 15935–15943
53. Henderson, J. P., Byun, J., Mueller, D. M., and Heinecke, J. W. (2001) Biochemistry 40, 2052–2059
54. Chen, H. J., Row, S. W., and Hong, C. L. (2002) Chem. Res. Toxicol. 15, 262–268
55. Whiteman, M., Jenner, A., and Halliwell, B. (1997) Chem. Res. Toxicol. 10, 1240–1246
56. Henderson, J. P., Byun, J., Williams, M. V., McCormick, M. L., Parks, W. C., Ridnour, L. A., and Heinecke, J. W. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 1631–1636
57. Henderson, J. P., Byun, J., Williams, M. V., Mueller, D. M., McCormick, M. L., and Heinecke, J. W. (2001) J. Biol. Chem. 276, 7867–7875
58. Henderson, J. P., Byun, J., Williams, M. V., Mueller, D. M., McCormick, M. L., and Heinecke, J. W. (2001) J. Biol. Chem. 276, 7867–7875
59. Lasken, R. S., and Goodman, M. F. (1984) J. Biol. Chem. 259, 11491–11495
60. Trautner, T. A., Swartz, M. N., and Kornberg, A. (1962) Proc. Natl. Acad. Sci. U. S. A. 48, 449–455
61. Brandon, M. L., Mi, L.-J., Chaung, W., Teebor, G., and Boorstein, R. J. (2000) Mutat. Res. 459, 161–169
62. Kubareva, E. A., Volkov, E. M., Vinogradova, N. L., Kanevsky, I. A., Oretskaya, T. S., Kuznetsova, S. A., Brevnov, M. G., Gromova, E. S., Nevinsky, G. A., and Shabarova, Z. A. (1995) Gene (Amst.) 157, 167–171
63. Baker, D., Liu, P. F., Burdzy, A., and Sowers, L. C. (2002) Chem. Res. Toxicol. 15, 33–39
64. Heartlein, M. W., O’Neill, J. P., and Preston, R. J. (1983) Mutat. Res. 107, 103–109
65. O’Neill, J. P., Heartlein, M. W., and Preston, R. J. (1983) Mutat. Res. 109, 259–270
66. Wilson, D. M., III, and Thompson, L. H. (2007) Mutat. Res. 616, 11–23