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Translesion Synthesis past Tamoxifen-derived DNA Adducts by Human DNA Polymerases η and κ[†]

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Abstract

The long-term treatment of tamoxifen (TAM), widely used for adjuvant chemotherapy and chemoprevention for breast cancer, increases a risk of developing endometrial cancer. A high frequency of K-*ras* mutations has been observed in the endometrium of women treated with TAM. Human DNA polymerase (pol) η and pol κ are highly expressed in the reproductive organs and associate with translesion synthesis past bulky DNA adducts. To explore the miscoding properties of α-(*N*^{2}-deoxyguanosinyl)tamoxifen (dG-N^2-TAM), a major TAM-DNA adduct, site-specifically modified oligodeoxynucleotides containing a single diastereoisomer of *trans*- or *cis*-forms of dG-N^2-TAM were prepared by phosphoramidite chemical procedure and used as templates. The primer extension reaction catalyzed by pol κΔC, a truncated form of pol κ, extended more efficiently past the adduct than that of pol η by incorporating dCMP, a correct base, opposite the adduct. With pol η, all diastereoisomers of dG-N^2-TAM promoted small amounts of direct incorporation of dAMP and deletions. With pol κΔC, dG-N^2-TAM promoted small amounts of dTMP and/or dAMP incorporations and deletion. The miscoding properties varied depending on the diastereoisomer of dG-N^2-TAM adducts and DNA pol used. Steady-state kinetic studies were also performed using either non-specific sequence or K-*ras* gene sequence containing a single dG-N^2-TAM at the second base of codon 12. With pol η, the bypass frequency past the dA•dG-N^2-TAM pair positioned in the K-*ras* sequence was only 2.3 times lower than that for the dC•dG-N^2-TAM pair, indicating that dG-N^2-TAM in K-*ras* sequence has higher miscoding potential than that in the non-specific sequence. However, with pol κΔC, the bypass frequency past the dC•dG-N^2-TAM pair was higher than that of the dT•dG-N^2-TAM pair in both sequences. The properties of pol η and pol κ are consistent with the mutagenic events attributed to TAM-DNA adducts.

Tamoxifen (TAM, the structure in Fig. 1) is widely used as a first-line endocrine therapy for breast cancer patients who are estrogen receptor positive and is also a prophylactic agent for preventing breast cancer in women at a high risk of this disease. Beside the significant benefit, long-term treatment of TAM in women increases the risk of developing endometrial cancer (1-5).

In rats, a high level of TAM-DNA adducts are produced in the liver (6,7) and initiate the development of hepatocellular carcinomas (8,9). TAM is converted to several α-hydroxylated metabolites which are in turn O-sulfonated by hydroxysteroid sulfotransferases and react with cellular DNA, resulting in the formation of four diastereoisomers [two *trans*-forms (fr-1 and fr-2) and two *cis*-forms (fr-3 and fr-4)] of α-(*N*^{2}-deoxyguanosinyl)tamoxifen (dG-N^2-TAM)

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and/or α-(*N*^{2}-deoxyguanosinyl)-*N*-desmethyltamoxifen (dG-N^2-N-desTAM) adducts (Fig. 1) (reviewed by 10). Among the diastereoisomers, a trans-form (fr-2) of dG-*N*^{2}-TAM and dG-N^2-N-desTAM were detected as a major TAM-DNA adduct in rodents (11-13) and monkey (14,15). TAM-DNA adducts were also detected in the endometrium of women treated with TAM (16-18) although there is some contradiction regarding the detection of TAM-DNA adducts in human tissues (19-22). A high frequency of K-*ras* mutations was recently observed in the endometrium of women treated with TAM (23); the mutational specificity was consistent with that occurred at the dG-N^2-TAM adduct (24). In addition to the genotoxic events, TAM has partial estrogenic activity through the estrogen receptor in endometrial cells and rat uterus (25). This effect may enhance in promoting cancer. Therefore, genotoxic damage and estrogenic activity may cause TAM-induced cancers.

Several human DNA polymerases (pol) are associated with translesion synthesis past DNA adducts (26,27). Among them, pol η and pol κ lack the 3'-5' exonuclease activity and as a result, copy DNA templates with low fidelity, thereby increasing the frequency of spontaneous mutations (28,29) and catalyzing miscoding reactions at several DNA lesions including UV-induced damages (30,31), 8-oxo-7,8-dihydro-deoxyguanosine (8-oxodG) (32) and 2-acetylaminofluorene (AAF)- (33) and 7,8-dihydroxy-anti-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE)-derived adducts (34). Pol κ also conducts translesion synthesis past abasic sites (35), 8-oxodG (36), 2-AAF- and BPDE-derived adducts (35-39). Both enzymes are highly expressed in human ovary and uterus (40-44) which are highly responsible for the production of steroidal hormones. The activated metabolites of endogenous and synthetic estrogens are suspected to form DNA adducts that are miscoded by such enzymes (40,41). Due to the structural similarity, the DNA pols may also miscode TAM-DNA adduct during translesion synthesis, increasing the risk of developing cancers in reproductive organs.

In the present study, site-specifically modified oligodeoxynucleotides containing a single diastereoisomer of dG-*N*^{2}-TAM were used as the DNA template for primer extension reactions catalyzed by pol η or a truncated form of human pol κ (pol κΔC). The miscoding properties of the TAM-DNA adducts were analyzed using an *in vitro* experimental system that quantifies base substitutions and deletions (45). A phosphoramidite chemical procedure established recently in our laboratory (46) makes it possible to prepare oligodeoxynucleotides containing a single diastereoisomer of dG-N^2-TAM adduct into the second base of codon 12 in K-*ras* gene sequence. Using the modified oligodeoxynucleotide as a template, steady-state kinetic studies were performed to determine the frequency of nucleotide insertion opposite the lesion and chain extension reactions for measuring the relative frequency of translesion synthesis past the dG-N^2-TAM adduct. Taken together, we found that dG-*N*^{2}-TAM is a miscoding adduct in reactions catalyzed by pol η and κ, suggesting the contribution of these enzymes to TAM mutagenicity.

MATERIALS AND METHODS

Synthesis of oligodeoxynucleotides

Oligodeoxynucleotides used as DNA template ($^{5'}$CCTTCXCTTCTTTCCTCTCCCTTT and $^{5'}$CATGCTGATGAATTCCTTCXCTTCTTTCCTCTCCCTTT; where X is G), primer and standard markers were prepared by solid-state synthesis on an automated DNA synthesizer. A 24-mer ($^{5'}$CCTTCXCTTCTTTCCTCTCCCTTT; where X is dG-N^2-TAM) containing a mixture of trans-forms (fr-1 and fr-2) of dG-N^2-TAM was prepared from the trans-dG-N^2-TAM phosphoramidite by chemical procedure established in our laboratory (46). The 24-mer oligomer containing fr-1 was isolated from that containing fr-2 by HPLC (46). Similarly, a 24-mer containing a mixture of cis-forms (fr-3 and fr-4) of dG-N^2-TAM was prepared from the cis-dG-N^2-TAM phosphoramidite and the oligomers containing fr-3 or fr-4 were separated by HPLC. The dG-N^2-TAM-modified 24-mer containing each diastereoisomer was then ligated

to a 14-mer ($^{5'}$CATGCTGATGAATT) to prepare the 38-mer template ($^{5'}$CATGCTGATGAATTCCTTCXCTTCTTTCCTCTCCCTTT; where X is dG-N^2-TAM) (47). The dG-N^2-TAM-modified and unmodified oligomers were purified on a Water's reverse-phase μBondapak C_{18} (0.39 × 30 cm), using a linear gradient of 0.05 M triethylammonium acetate, pH 7.0, containing 10 - 50% acetonitrile with an elution time of 60 min and a flow rate of 1.0 mL/min (24). The oligomers were further purified by electrophoresis on 20% polyacrylamide denaturing gel (PAGE) (35 × 42 × 0.04 cm) (48). Oligomers were labeled at the 5' terminus by treating with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP (49) and subjected to 20% PAGE containing 7M urea (35 × 42 × 0.04 cm) to establish homogeneity. The position of the oligomers was established by β-phosphorimager analysis (Molecular Dynamics Inc.).

Primer extension reactions

Pol η and a truncated form of pol κ (pol κΔC) were prepared as described previously (30,35). Although pol κΔC has a lower processivity than the full-length pol κ, the miscoding rate on the undamaged DNA by pol κΔC was similar to that of pol κ (28). A 10-mer ($^{5'}$AGAGGAAAGA) or 12-mer ($^{5'}$AGAGGAAAGAAG) was labeled at the 5' terminus with T4 polynucleotide kinase and [γ-^{32}P]-ATP (49). Using dG-N^2-TAM-modified or unmodified 38-mer oligodeoxynucleotide (200 fmol) primed with a ^{32}P-labeled 10-mer ($^{5'}$AGAGGAAAGA; 100 fmol) or 12-mer ($^{5'}$AGAGGAAAGAAG; 100 fmol), primer extension reactions catalyzed by pol η or pol κΔC were conducted at 25°C for 30 min in a buffer (10 μL) containing four dNTPs (100 μM each) (Fig. 2). The reaction buffer for pol η contains 40 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$, 10 mM DTT, 250 μg/mL BSA, 60 mM KCl, and 2.5 % glycerol. A similar reaction buffer was used for pol κΔC, using 5 mM $MgCl_2$ instead of 1 mM $MgCl_2$. Reactions were stopped with the addition of 5 μL of formamide dye. The samples were subjected to 20% PAGE (35 × 42 × 0.04 cm). The radioactivity of extended products was measured by β-phosphorimager (Molecular Dynamics).

Miscoding Analysis

Using dG-N^2-TAM-modified or unmodified 38-mer oligodeoxynucleotide (450 fmol) primed with a ^{32}P-labeled 12-mer ($^{5'}$AGAGGAAAGTAG; 300 fmol), primer extension reactions catalyzed by pol η or pol κΔC (15 ng for the unmodified template; 150 ng for the modified templates) were conducted at 25°C for 30 min in a buffer (30 μl) containing four dNTPs (100 μM each) and subjected to 20% PAGE (35 × 42 × 0.04 cm). Extended reaction products (approximately 28−32 bases long) were extracted from the gels. The recovered oligodeoxynucleotides were annealed with an unmodified 38-mer, cleaved with *Eco* RI, and subjected to electrophoresis on two-phase 20% polyacrylamide gels (15 × 72 × 0.04 cm) containing 7M urea in the upper phase and no urea in the lower phase (two-phase PAGE) (45) (Fig. 2). To quantify base substitutions and deletions, mobility of the reaction products were compared with those of 18-mer standards containing dC, dA, dG or dT opposite the lesion and one (Δ^1) or two-base (Δ^2) deletions. The detection limit on the gel was 0.03% of the labeled oligomers.

Steady-state kinetic studies

Kinetic parameters associated with nucleotide insertion opposite dG-N^2-TAM lesion and chain extension from the 3' primer terminus, were determined at 25 °C, using varying amounts of single dNTPs (0 - 500 μM). For insertion kinetics, reaction mixtures containing dNTP (0 - 500 μM) and either pol η (0.5 - 5 ng) or pol κΔC (0.5 - 5 ng) were incubated at 25 °C for 2 min in 10 μL of Tris-HCl buffer (pH 8.0) using a 24-mer template (150 fmol; $^{5'}$CCTTCXCTTCTTTCCTCTCCCTTT, where X is G or dG-N^2-TAM) primed with a ^{32}P-labeled 12-mer primer (100 fmol; $^{5'}$AGAGGAAAGAAG). Reaction mixtures containing

a template (150 fmol) primed with a ^{32}P-labeled 13-mer primer (100 fmol; 5'AGAGGAAAGAAGN, where N is C, A, G or T), with varying amounts of dGTP (0 - 500 μM), and either pol η (0.5 - 5 ng) or pol κΔC (0.5 - 5 ng) were used to measure chain extension. Another 21-mer template containing a codon 12 of K-*ras* sequence [150 fmol; 5'GGAGCTGXTGGCGTAGGCTGT (codons 10−16), where X is G or dG-N^2-TAM] was also used for determining the frequency of dNTP insertion opposite the lesion using a ^{32}P-labeled 13-mer primer (100 fmol; 5'ACAGCCTACGCCA). Reaction mixtures containing a template (150 fmol) primed with a ^{32}P-labeled 14-mer primer (100 fmol; 5'ACAGCCTACGCCAN, where N is C, A, G or T), with varying amounts of dCTP (0 - 500 μM), and either pol η (0.5 - 5 ng) or pol κΔC (0.5 - 5 ng) were used to measure chain extension. The reaction samples were subjected to 20% denaturing PAGE (35 × 42 × 0.04 cm). The Michaelis constants (K_m) and maximum rates of reaction (V_{max}) were obtained from Hanes-Woolf plots. Frequencies of dNTP insertion (F_{ins}) and chain extension (F_{ext}) were determined relative to the dC•dG base pair according to the equation: $F = (V_{max}/K_m)_{[wrong\ pair]}/(V_{max}/K_m)_{[correct\ pair=dC•dG]}$ (50,51).

RESULTS

Miscoding properties of dG-N^2-TAM DNA adducts in reactions catalyzed by pol η

Primer extension reactions catalyzed by pol η were conducted on unmodified or dG-N^2-TAM-modified 38-mer template in the presence of four dNTPs (Fig. 3). On the templates containing a single unmodified dG, primer extensions catalyzed by this enzyme readily occurred to form the extended products. With the modified templates containing a single diastereoisomer (fr-1, fr-2, fr-3 and fr-4) of dG-N^2-TAM adduct, primer extensions were retarded one-base prior to the adduct, opposite the adduct, and one-base past the adduct. The extended products past the dG-N^2-TAM lesions were obtained by increasing the amount of the enzyme. When 500 fmol of pol η was used, approximately 4.5 ± 1.0 % for fr-1, 47 ± 3.4 % for fr-2, 33 ± 2.6 % for fr-3, and 17 ± 1.9 % for fr-4 of the starting primer were extended past the adducts respectively to form the fully-extended products (28−34 mers). Blunt-end addition to the fully extended product (33−34 mers) was observed (52,53). The primer extension past the fr-2 or fr-3 was more efficient than that of fr-1 and fr-4, indicating that the frequency past the adducts varied depending on the diastereoisomers.

The fully extended products (approximately 28−34-mers) past the unmodified or modified adducts were recovered, digested by *Eco* RI, and subjected to two-phase PAGE for quantitative analysis of base substitutions and deletions (Fig. 2). A standard mixture of six ^{32}P-labeled oligodeoxynucleotides containing dC, dA, dG, or dT opposite the adduct, and containing one- and two-base deletions can be resolved by this method (Fig. 4). Preferential incorporation of dCMP, the correct bases, opposite the unmodified dG was observed. As indicated by arrows, small amounts of unknown products were detected; the migration of these products differed from those of standard markers. Since the amount of the fully extended products past the dG-N^2-TAM were less than that past the unmodified dG, the fully extended products past the each diastereoisomer of dG-N^2-TAM were recovered from several reactions and used for the two-phase PAGE analysis (Fig. 4). The amounts of dNMP incorporation opposite each diastereoisomer were normalized for the amounts of their starting primer. dG-N^2-TAM promoted preferential incorporation of dCMP (4.3 % for fr-1; 32.0 % for fr-2; 22.5 % for fr-3; 13.8 % for fr-4, respectively) opposite the adducts. A small amount of dAMP incorporation (0.04 % for fr-1; 1.2 % for fr-2; 2.3 % for fr-3; 1.1 % for fr-4) opposite the adduct was observed, in addition to significant amounts of one-base (0.08−2.4 %) and two-base (0.06−9.9 %) deletions. As observed for the unmodified dG, small amount of unknown products were observed.

Miscoding properties of dG-N^2-TAM adducts in reactions catalyzed by pol κΔC

Primer extension reactions catalyzed by pol κΔC readily occurred on the templates containing unmodified dG to form the extended products (Fig. 3). When 10 and 100 fmol of this enzyme were used, 68 % and 92 %, respectively, of the primer were extended past dG to form the fully extended products. Unlike pol η, the primer extension reactions were retarded one-base prior to the fr-1 of dG-N^2-TAM and opposite the adduct; with 100 fmol of pol κΔC, only 9.0 % of primers were extended past this adduct to form the fully extended products. However, using the same amount of enzyme, majority of primers (81−90 %) were readily extended past the fr-2, fr-3 or fr-4 of dG-*N*^{2}-TAM to form the fully extended products.

The extended products past the unmodified and modified adducts were used for miscoding analysis using two-phase PAGE. When the unmodified template was used, an expected incorporation of dCMP was observed opposite the dG (Fig. 4). All diastereoisomers of dG-N^2-TAM promoted incorporation of dCMP (36.7 % for fr-1; 83.3 % for fr-2; 82.3 % for fr-3; 72.7 % for fr-4, respectively) as a primary product. When the fr-1 was used, small amount of dAMP (0.7 %) and dTMP (0.55 %) incorporation and one-base deletion (1.0 %) were observed at the adduct. With other diastereoisomers (fr-2, fr-3 and fr-4), only a small amount of dTMP incorporation (0.6 % for fr-2; 1.8 % for fr-3; 1.4 % for fr-4) was observed.

Kinetic studies on dG-N^2-TAM-modified DNA templates

The frequency of dNTP incorporation (F_{ins}) opposite a trans-form (fr-2) of dG-N^2-TAM and the frequency of chain extension (F_{ext}) from dN•dG-N^2-TAM pairs were measured within the linear range of the reaction, using the same sequence context that was used for the two-phase PAGE assay (Table 1). With pol η, the F_{ins} value for dCTP (2.45×10^{-2}), the correct base, opposite the dG-N^2-TAM was only 1.7 times higher than that of dATP (1.44×10^{-2}) and was 13 and 41 times higher than that of dGTP and dTTP, respectively. Since the F_{ext} for the dC•dG-N^2-TAM pair was 36 times higher than that for the dA•dG-N^2-TAM pair, the relative bypass frequency ($F_{ins} \times F_{ext}$) past the dC•dG-N^2-TAM pair was approximately 62 times higher than that for the dA•dG-N^2-TAM pair. The $F_{ins} \times F_{ext}$ past the dC•dG-N^2-TAM pair was two to three orders of magnitude higher than that of the other dN•dG-N^2-TAM pairs. When pol κΔC was used, the F_{ins} for dCTP opposite the dG-*N*^{2}-TAM was 11 and 35 times higher than that of dTTP and dATP, respectively (Table 1). The F_{ins} for dGTP was not detected. The $F_{ins} \times F_{ext}$ for the dC•dG-N^2-TAM pair was 50 and 940 times higher than that for the dT•dG-N^2-TAM and dA•dG-*N*^{2}-TAM pairs, respectively.

Steady-state kinetic studies were also performed using K-*ras* sequence containing a single dG-N^2-TAM (fr-2) positioned at the second base of codon 12 (Table 1). With pol η, the F_{ins} value for dCTP opposite the dG-N^2-TAM was 2.7, 5.6 and 13 times higher than that of dATP, dGTP and dTTP, respectively. Interestingly, the F_{ext} for dA•dG-N^2-TAM pair (3.37×10^{-3}) was slightly higher than that of the dC•dG-N^2-TAM pair (2.85×10^{-3}), resulting that the $F_{ins} \times F_{ext}$ for dA•dG-N^2-TAM pair was only 2.3 times lower than that of the dC•dG-N^2-TAM pair. The $F_{ins} \times F_{ext}$ for dC•dG-N^2-TAM pair was 45 and 3100 times higher than that of dG•dG-N^2-TAM and dT•dG-N^2-TAM pairs, respectively. When pol κΔC was used, the F_{ins} for dCTP and dTTP were only detected; the F_{ins} for dTTP was 48 times lower than that of dCTP. The $F_{ins} \times F_{ext}$ for dT•dG-*N*^{2}-TAM was 430 times lower than that of the dC•dG-*N*^{2}-TAM pair.

DISCUSSION

Pol η and pol κ are highly expressed in the reproductive organs including ovary and uterus (40-44) where steroidal hormones are produced. Since TAM has a partial structure of estrogen, these DNA pols may contribute to translesion synthesis past DNA adducts derived from TAM. In fact, the primer extension reactions catalyzed by pol κΔC occurred more rapidly past the

dG-N^2-TAM adduct than that of pol η by incorporating preferentially dCMP, the correct base, opposite the adduct. With pol η, a small amount of direct dAMP incorporation was observed with deletions, indicating that G → T mutations and deletions were produced. With pol κΔC, dG-N^2-TAM promoted small amounts of dTMP incorporations, indicating that G → A mutations occurred. This observation was supported by steady-state kinetic studies. The miscoding specificities detected with these enzymes were consistent with mutagenic events induced by dG-N^2-TAM in a single-strand vector propagated in simian kidney (COS-7) cells (24).

Unlike other bulky DNA adducts (38,39,54), dG-N^2-TAM allowed pol α, β, or δ, in addition to pol η and κ, to extend past the adduct (47). Pol α promoted the misincorporation of dAMP and deletions at all the diastereoisomers, accompanied by small amounts of dGMP incorporation. Pol β promoted deletions and misincorporation of dAMP and dGMP. With pol δ, preferential incorporation of dCMP was observed: fr-1 of dG-N^2-TAM only promoted a small amount of dTMP incorporation. Some of these replicative DNA pols may also be involved in the miscoding events induced by TAM. Thus, the miscoding specificities and frequencies varied depending on the DNA polymerase used.

Among four diastereisomers [two trans-forms (fr-1 and fr-2) and two cis-forms (fr-3 and fr-4)], the primer extension reactions catalyzed by pol η and pol κΔC were retarded strongly at the fr-1; therefore, with pol η, formation of dCMP and dAMP incorporations and deletions at the fr-1 was much lower than that observed with other diastereoisomers. With pol κΔC, small amounts of dAMP and dTMP incorporations and one-base deletion were observed opposite the fr-1 while a small amount of dTMP incorporation was only observed with other diastereoisomers. Thus, the miscoding specificity and frequency also varied depending on the diastereoisomer of dG-N^2-TAM adduct. The confirmation of each diastereoisomer in the replication folk may affect the miscoding properties, as suggested by the three dimensional NMR studies (55).

As observed with COS-7 cells (24) and with pol η and κ in the present study, a high frequency of G → T and G → A mutations were detected at the second base in K-*ras* codon 12 in the endometrium of women treated with TAM (23). A phosphoramidite chemical synthesis established in our laboratory has made it possible to insert dG-N^2-TAM into the oligomers having any sequence context such as K-*ras* gene. The frequencies of dNTP insertion opposite the dG-N^2-TAM and the chain extension from the dN•dG-N^2-TAM pair were determined using the K-*ras* sequence and compared it with those observed with non-specific sequence. Since a trans-form (fr-2) of dG-N^2-TAM was a major TAM-DNA adducts in rodents (11-13) and primate (14,15), this isomer was used for this kinetic studies. With pol η, the $F_{ins}\times F_{ext}$ for the dA•dG-N^2-TAM pair in the K-*ras* sequence was only 2.3 times lower than that of the dC•dG-N^2-TAM pair while, with the non-specific sequence, the $F_{ins}\times F_{ext}$ for dA•dG-N^2-TAM pair in the K-*ras* sequence was 62 times lower than that of the dC•dG-N^2-TAM pair (Table 1). This indicated that specific sequence context such as K-*ras* gene may affect to increase the miscoding frequency, resulting in the construction of mutational hot-spots.

Since G → A mutations also were observed in codon 12 of K-*ras* gene in the endometrium of women treated with TAM (23), some of DNA pols are expected to insert dTMP opposite the dG-N^2-TAM lesion. Only pol κ has this ability among DNA pols examined. Although with pol κΔC, the $F_{ins}\times F_{ext}$ past the dT•dG-N^2-TAM was 50 times lower than that of dC•dG-N^2-TAM pair, a low level of direct dTMP incorporation was detected during translesion synthesis catalyzed by this enzyme (Fig. 4). Since pol ι is highly expressed in the reproductive organs (44,56) and tends to incorporate dTMP opposite the bulky DNA adducts (27), this enzyme may contribute to the mutagenic events of TAM-DNA adducts, resulting in the increase of G → A mutations.

To compare the behavior of pol η or pol κ past the dG-N^2-TAM adduct with that past several bulky dG-N^2 adducts such as dG-N^2-3MeE, (+)-*trans*-dG-N^2-BPDE, or dG-N^2-AAF, these adducts were embedded in a similar sequence context (39,54,57) (Table 2). With pol η, like dC•dG-N^2-AAF and dC•dG-N^2-3MeE pairs, the $F_{ins}\times F_{ext}$ for the dC•dG-N^2-TAM pair was one to four orders of magnitude higher than that of any of the nucleotides paired with dG-N^2-TAM. Interestingly, the $F_{ins}\times F_{ext}$ ratio for dC•dG-N^2-TAM/dA•dG-N^2-TAM pair was 62 and that number was higher than that for the dC•dG-N^2-3MeE/dA•dG-N^2-3MeE (310) or dC•dG-N^2-AAF/dA•dG-N^2-AAF (165) pair, indicating that dG-N^2-TAM adduct may have higher miscoding potential than dG-N^2-3MeE or dG-N^2-AAF. Pol κ bypassed dG-N^2-TAM adduct more efficiently by incorporating dCMP opposite the lesion, as observed with dG-N^2-3MeE, (+)-*trans*-dG-N^2-BPDE, or dG-N^2-AAF lesion (Table 2). However, the $F_{ins}\times F_{ext}$ ratio for dC•dG-N^2-TAM/dT•dG-N^2-TAM pair was 50 and that number was one to three orders of magnitude higher than that for the dC•dG-N^2-3MeE/dT•dG-N^2-3MeE (5400) or dC•(+)-*trans*-dG-N^2-BPDE/dT•(+)-*trans*-dG-N^2-BPDE (21000), or dC•dG-N^2-AAF/dT•dG-N^2-AAF (210) pair, indicating that dG-N^2-TAM adduct may also have higher miscoding potential than other bulky DNA adducts. Pol κ and pol η may have evolved to bypass dG-N^2-TAM.

In conclusion, human DNA pols η and κ expressed highly in reproductive organs are more likely to be associated with miscoding events generated by TAM. The high frequency of mutations observed at the K-*ras* sequence may reflect the sequence context effect. Thus, dG-N^2-TAM adducts generate mutations and pose a potential risk to women treated with TAM. This result raises concerns about the use of TAM as a chemopreventive agent for healthy women as well as its use in treatment of breast cancer.

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1Abbreviations

dNTP, 2'-deoxynucleoside triphosphate; TAM, tamoxifen; dG-N^2-TAM, α-(N^2-deoxyguanosinyl)tamoxifen; pol η, DNA polymerase η; pol κ, DNA polymerase κ; pol κΔC, a truncated form of pol κ; F_{ins}, frequency of insertion; F_{ext}, frequency of extension; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography..

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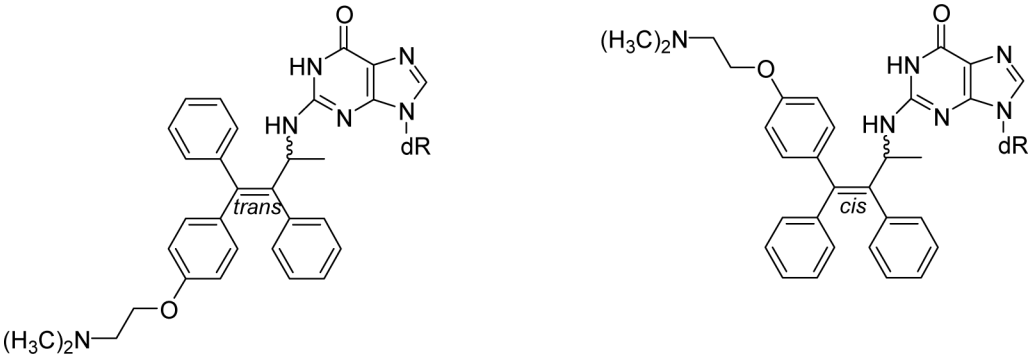

Fig. 1.
Structures of dG-N^2-TAM adduct.

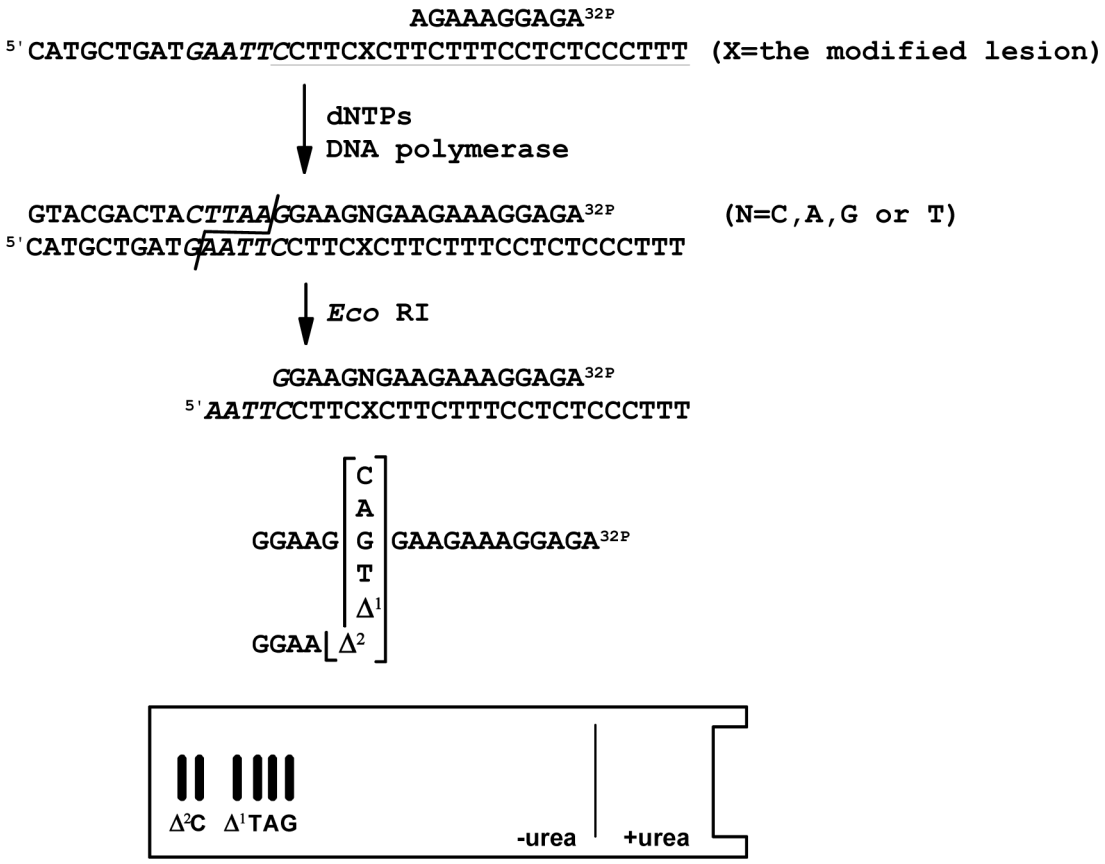

Fig. 2. Diagram of the method used to determine miscoding specificities

Unmodified or dG-N^2-TAM-modified 38-mer templates are annealed to a ^{32}P-labeled 10-mer primer. Primer extension reactions catalyzed by pol η or pol κΔC were conducted in the presence of four dNTPs. Fully extended products formed during DNA synthesis are recovered from the polyacrylamide gel (35 × 42 × 0.04 cm), annealed with a complementary 38-mer, cleaved with *Eco*RI, and subjected to a two-phase PAGE (15 × 72 × 0.04 cm), as described in Materials and Methods. To determine miscoding specificity, mobility of the reaction products are compared with those of 18-mer standards containing dC, dA, dG or dT opposite the lesion and one (Δ^1) or two-base (Δ^2) deletions.

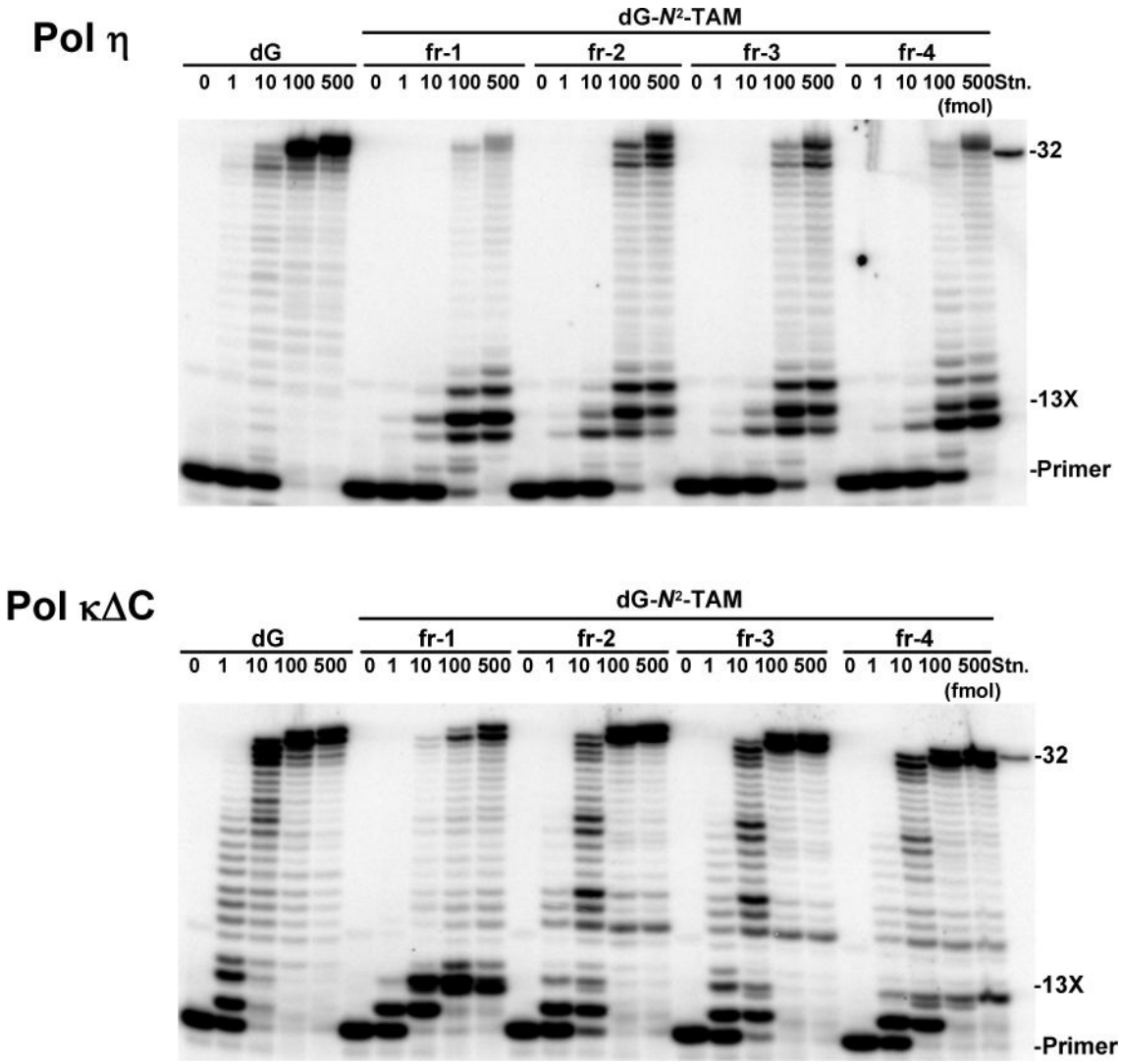

Fig. 3. Primer extension reactions catalyzed by pol η or pol κΔC on the dG-N^2-TAM-modifiedtemplates

Using unmodified or dG-N^2-TAM-modified 38-mer templates primed with a ^{32}P-labeled 10-mer (^{32P}-AGAGGAAAGA), primer extension reactions were conducted at 25°C for 30 min in a buffer containing four dNTPs (100 μM each) and variable amounts (0, 1.0, 10, 100, and 500 fmol) of pol η or pol κΔC as described in Materials and Methods. One-third of the reaction mixture was subjected to PAGE (35 × 42 × 0.04 cm). The radioactivity of extended products was measured by β-phosphorimager. 13X shows the location opposite the dG-N^2-TAM lesion. A 32-mer ($^{5'}$AGAGGAAAGAAGCGAAGGAATTCATCAGCATG) was used as a marker of fully extended product (Stn.).

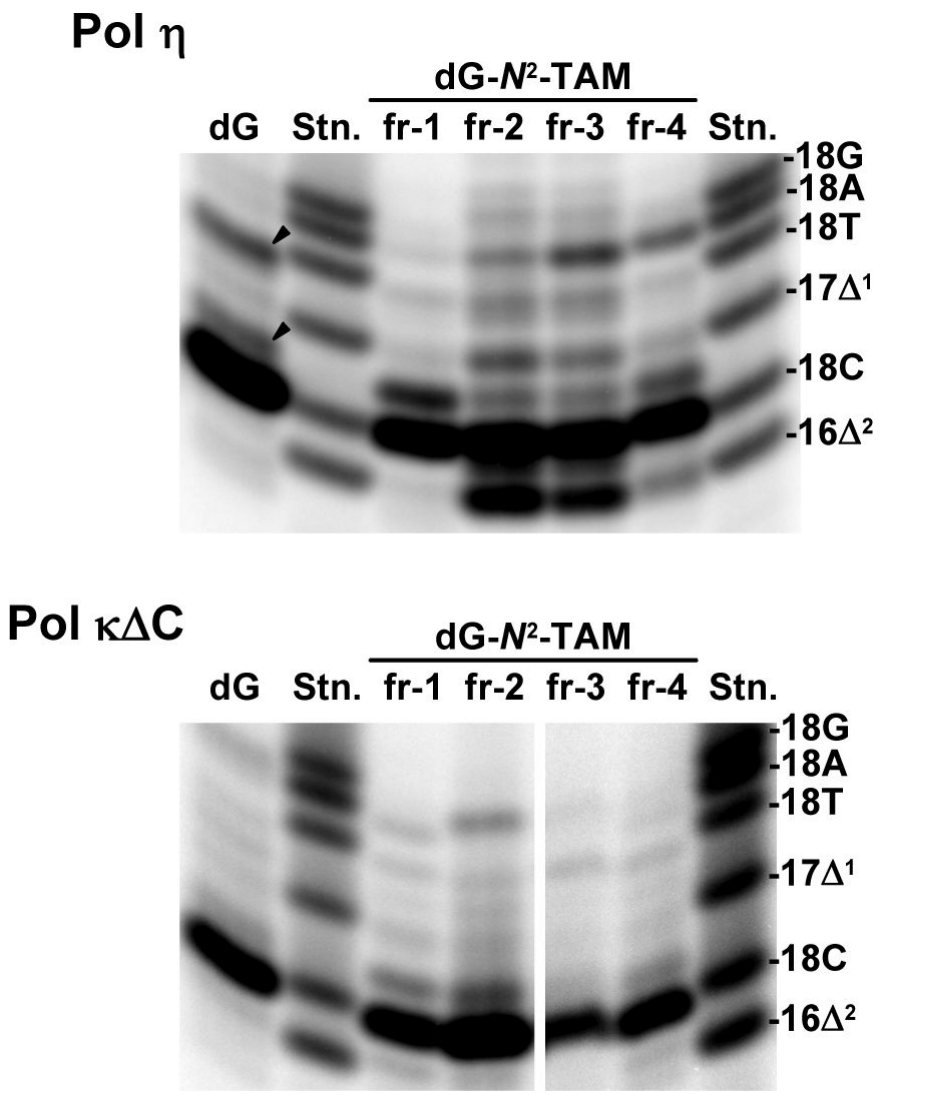

Fig. 4. Quantitation of miscoding specificities induced by dG-N^2-TAM adduct

A 12-mer primer (^{32P}-AGAGGAAAGAAG) was used for the primer extension reactions catalyzed by 100 fmol of pol η or pol κΔC, as described in Fig. 3. Three each independent reaction samples were combined and subjected to PAGE (35 × 42 × 0.04 cm). The fully-extended products recovered from the gel were used for analysis of base substitutions and deletions, as described in the legend of Fig. 2.

Table 1

Kinetic Parameters for Nucleotide Insertion and Chain Extension Reactions Catalyzed by DNA pol η and pol κ[a]

Insertion dNTP ↓GAAGAAAGGAGA^{32P} 5'CCTTCXCTTCTTTCCTCTCCCTTT				Extension dGTP ↓NGAAGAAAGGAGA^{32P} 5'CCTTCXCTTCTTTCCTCTCCCTTT			
N:X	**K_m (μM)**	**V_{max}(%min^{-1})**	**F_{ins}**	**K_m (μM)**	**V_{max}(%min^{-1})**	**F_{ext}**	**F_{ins}×F_{ext}**
Pol η							
X = dG							
C:G	0.87± 0.08[b]	47.8 ± 0.19[b]	**1.0**	0.47± 0.12[b]	54.8 ± 4.1[b]	**1.0**	**1.0**
X = dG-N^2-TAM							
C:X	2.44 ± 0.04	3.28 ±0.39	**2.45×10^{-2}**	7.61 ± 0.53	5.05 ± 0.45	**5.69×10^{-3}**	**1.39×10^{-4}**
A:X	22.5 ± 1.0	17.8 ± 0.09	**1.44×10^{-2}**	13.1 ± 2.4	0.24 ± 0.03	**1.57×10^{-4}**	**2.26×10^{-6}**
G:X	23.2 ± 1.4	2.37 ± 0.01	**1.86×10^{-3}**	14.3 ± 1.4	0.23 ± 0.01	**1.38×10^{-4}**	**2.57×10^{-7}**
T:X	69.5 ± 6.2	2.31 ±0.07	**6.05×10^{-4}**	16.7 ± 1.9	0.08 ± 0.01	**4.10×10^{-5}**	**2.48×10^{-8}**
Pol κΔC							
X = dG							
C:G	1.68 ± 0.23[b]	39.1 ± 3.46[b]	**1.0**	1.61 ± 0.08[b]	111.0 ± 7.7[b]	**1.0**	**1.0**
X = dG-N^2-TAM							
C:X	12.5 ± 0.1	5.47 ± 0.01	**1.88×10^{-2}**	5.06 ± 1.17	2.42 ± 0.01	**6.94×10^{-3}**	**1.30×10^{-4}**
A:X	32.3 ± 6.4	0.41 ± 0.02	**5.45×10^{-4}**	33.9 ± 0.7	0.59 ± 0.01	**2.53×10^{-4}**	**1.38×10^{-7}**
G:X	N.D.	N.D.	**N.D.**	115 ± 26	1.21 ± 0.10	**1.53×10^{-4}**	**N.D.**
T:X	19.3 ± 2.2	0.80 ± 0.11	**1.78×10^{-3}**	6.65 ± 1.06	0.67 ± 0.01	**1.46×10^{-3}**	**2.60×10^{-6}**

Insertion dNTP ↓ACCGCATCCGACA^{32P} 5'GGAGCTGXTGGCGTAGGCTGT				Extension dCTP ↓NACCGCATCCGACA^{32P} 5'GGAGCTGXTGGCGTAGGCTGT			
N:X	**K_m (μM)**	**V_{max}(%min^{-1})**	**F_{ins}**	**K_m (μM)**	**V_{max}(%min^{-1})**	**F_{ext}**	**F_{ins}×F_{ext}**
Pol η							
X = dG							
C:G	0.82 ± 0.13[b]	436 ± 27[b]	**1.0**	0.33 ± 0.10[b]	260 ± 4.8[b]	**1.0**	**1.0**
X = dG-N^2-TAM							
C:X	3.99 ± 0.69	2.93 ± 0.02	**1.50×10^{-3}**	0.85 ± 0.17	1.97 ± 0.01	**2.85×10^{-3}**	**4.26×10^{-6}**
A:X	8.18 ± 0.1	2.47 ± 0.04	**5.59×10^{-4}**	0.88 ± 0.19	2.42 ± 0.10	**3.37×10^{-3}**	**1.89×10^{-6}**
G:X	17.9 ± 0.8	2.57 ± 0.09	**2.66×10^{-4}**	3.94 ± 0.22	1.17 ± 0.01	**3.58×10^{-4}**	**9.52×10^{-8}**
T:X	29.1 ± 5.5	1.88 ± 0.16	**1.20×10^{-4}**	6.26 ± 0.86	0.06 ± 0.01	**1.15×10^{-5}**	**1.38×10^{-9}**
Pol κΔC							
X = dG							
C:G	2.31 ± 0.22[b]	136 ± 18[b]	**1.0**	1.02 ± 0.03[b]	127 ± 3[b]	**1.0**	**1.0**
X = dG-N^2-TAM							
C:X	11.6 ± 2.0	3.24 ± 0.03	**4.80×10^{-3}**	3.41 ± 0.83	1.11 ± 0.01	**2.69×10^{-3}**	**1.29×10^{-5}**
A:X	N.D.	N.D.	**N.D.**	5.62 ± 0.21	0.94 ± 0.01	**1.34×10^{-3}**	**N.D.**
G:X	N.D.	N.D.	**N.D.**	31.8 ± 9.4	2.84 ± 0.16	**7.42×10^{-4}**	**N.D.**
T:X	34.6 ± 3.4	0.21 ± 0.01	**1.01×10^{-4}**	32.2 ± 2.01	1.19 ± 0.06	**2.95×10^{-4}**	**2.99×10^{-8}**

[a] Kinetics of nucleotide insertion and chain extension reactions were determined as described in Materials and Methods. Frequencies of nucleotide insertion (F_{ins}) and chain extension (F_{ext}) were estimated by the equation: $F = (V_{max}/K_m)$[wrong pair]/(V_{max}/K_m) [correct pair =dC:dG]. X = dG or dG-N^2-TAM lesion.

[b] Data expressed as mean ± S.D. obtained from three independent experiments.

Table 2

$F_{ins} \times F_{ext}$ Past DNA Adducts by pol η or pol κ.

		dG-N^2-TAM	dG-N^2-3MeE[a]	dG-N^2-AAF[b]	(+)*trans*-dG-N^2-BPDE[c]
pol η	C:X	1.39×10^{-4}	9.50×10^{-4}	1.53×10^{-3}	—
	A:X	2.26×10^{-6}	3.06×10^{-6}	9.34×10^{-7}	—
	G:X	2.57×10^{-7}	2.50×10^{-6}	3.24×10^{-10}	—
	T:X	2.48×10^{-8}	3.99×10^{-6}	1.70×10^{-7}	—
pol κ	C:X	1.30×10^{-4}	1.33×10^{-1}	1.05×10^{-3}	1.09×10^{-5}
	A:X	1.38×10^{-7}	1.12×10^{-6}	7.35×10^{-9}	3.37×10^{-10}
	G:X	N.D.	4.30×10^{-7}	1.07×10^{-8}	8.24×10^{-12}
	T:X	2.60×10^{-6}	2.48×10^{-5}	4.90×10^{-6}	5.20×10^{-10}

[a] Data are taken from ref 54.

[b] Data are taken from ref 57.

[c] Data are taken from ref 39. N.D.; not detectable.