# Mutation Spectra Induced by α-Acetoxytamoxifen−DNA Adducts in Human DNA Repair Proficient and Deficient (Xeroderma Pigmentosum Complementation Group A) Cells†

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ABSTRACT: Tamoxifen, a breast cancer drug, has recently been approved for the chemoprevention of this disease. However, tamoxifen causes hepatic carcinomas in rats through a genotoxic mechanism and increases the risk of endometrial tumors in women. DNA adducts have been detected at low levels in human endometrium, and there is much interest in determining whether DNA damage plays a role in tamoxifen-induced endometrial carcinogenesis. This study investigates the mutagenicity of tamoxifen DNA adducts formed by α-acetoxytamoxifen, a reactive ester producing the major DNA adduct formed in livers of tamoxifen-treated rats. pSP189 plasmid DNA containing the *supF* gene was treated with α-acetoxytamoxifen and adduct levels (0.5−8.0 adducts per plasmid) determined by $^{32}$P-postlabeling. Adducted plasmids were transfected into nucleotide excision repair proficient (GM00637) or deficient (GM04429, *XPA*) human fibroblasts. After replication, plasmids were recovered and screened in indicator bacteria. Relative mutation frequencies increased with the adduct level, with 1.3−3.6-fold higher numbers of mutations in the XP cells compared to the GM00637 cells, indicating that NER plays a significant role in the removal of these particular tamoxifen DNA adducts. The majority of sequence alterations (91−96%) occurred at GC base pairs, as did mutation hotspots, although the type and position of mutations was cell-specific. In both cell lines, as the adduct level increased, the proportion of GC → AT transitions decreased and GC → TA transversions, mutations known to arise from the major tamoxifen adducts, increased. Given the high mutagenicity of dG-$N^2$-tamoxifen adducts, if not excised, they may potentially contribute to the initiation of endometrial cancer in women.

The antioestrogenic drug tamoxifen is widely used in the adjuvant therapy of breast cancer. On the basis of the finding that tamoxifen achieved a 49% reduction in the incidence of invasive breast cancer in healthy women (*1*), it has recently been approved by the United States Food and Drug Administration (FDA) for use as a chemopreventative agent in women at high risk of breast cancer. However, a concern over the use of this drug, particularly in healthy women, comes from the fact that tamoxifen treatment has been shown to increase the risk of endometrial cancer by 2−7-fold relative to controls (*2*, *3*). Administration of tamoxifen to rats results in the development of hepatocellular carcinomas via a genotoxic mechanism associated with the formation of numerous hepatic DNA adducts (*4*−*6*). Low levels of tamoxifen DNA adducts have also been detected in endometrial tissue and leukocytes of women undergoing tamoxifen therapy (*7*−*9*). Furthermore, binding of [$^{14}$C]tamoxifen to endometrial DNA has been demonstrated by accelerator mass spectrometry, following treatment of women with a single $^{14}$C-labeled therapeutic dose (*10*), which highlights the need to understand the consequences of tamoxifen DNA adduct formation in human cells. This is however a contentious issue because other investigators have failed to find evidence of tamoxifen DNA adduct formation in endometrial tissue of treated patients, using both $^{32}$P-postlabeling (*11*, *12*) and electrospray mass spectrometry analysis (*13*). Reported differences in the level of tamoxifen adducts in uterine samples from women receiving tamoxifen, particularly an absence of detectable adducts in half of the patients examined in one study (*8*), have been suggested as being due to individual variability in the extent of metabolic activation or DNA repair capacity. Factors influencing the repair of specific tamoxifen adducts may therefore affect the mutagenic potential of this drug in human endometrium.

Formation of tamoxifen adducts in rat liver requires activation of the parent drug or other phase I metabolites, which principally involves α-hydroxylation (*14*−*17*) followed by hydroxysteroid sulfotransferase-mediated sulfate conjugation (*18*, *19*). The carbocation arising from the loss of the sulfate moiety reacts predominantly with the $N^2$ amino group of deoxyguanosine and can form four diastereoisomers

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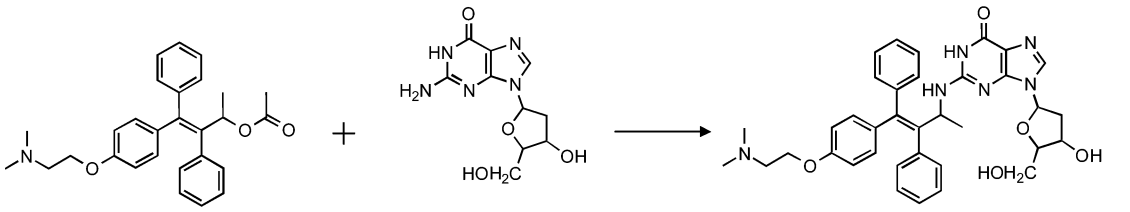


FIGURE 1: α-Acetoxytamoxifen reacts with deoxyguanosine to form the dG-$N^2$-tamoxifen adduct.

of α-(deoxyguanosin-$N^2$-yl)tamoxifen (dG-$N^2$-tamoxifen)[1] (*20*, *21*). A model compound, α-acetoxytamoxifen, is often used as a reactive derivative of tamoxifen for *in vitro* studies, because it has a longer half-life than the α-sulfate ester and when incubated with DNA yields the same dG-$N^2$-tamoxifen adducts that are formed in rats and humans after treatment with tamoxifen (*8*, *22*) (Figure 1).

Studies in COS-7 cells have shown that single site-specific dG-$N^2$-tamoxifen adducts induce primarily GC → TA transversions, with smaller amounts of GC → AT transitions (*23*). In addition, administration of tamoxifen causes GC → TA transversions in both the *cII* and *lacI* genes in *λ*/*lacI* transgenic rats (*24*, *25*). We have also recently demonstrated that adducts formed by α-acetoxytamoxifen preferentially induce GC → TA transversions in the *Escherichia coli supF* gene when replicated in human kidney cells (*26*), with an equivalent mutagenicity to the classic mutagen, benzo[*a*]-pyrene diol epoxide (BPDE). Bulky DNA adducts, such as those formed by tamoxifen, are typically removed by the nucleotide excision repair (NER) system. The enzymes involved in NER were first discovered in cells of patients with the genetic disease xeroderma pigmentosum (XP). Sufferers of this disease have a high incidence of skin cancers in areas of their bodies exposed to the sun because of impaired ability to repair UV-induced DNA damage (*27*), and fibroblast cells derived from these patients are deficient in nucleotide excision repair enzymes. To assess the importance of NER in the mutagenesis of tamoxifen-induced DNA damage, we have compared the mutation frequency and spectra occurring when α-acetoxytamoxifen-adducted shuttle vector plasmid, containing the *supF* gene (*28*), was replicated in repair-deficient human fibroblast cells from a patient with xeroderma pigmentosum (GM04429, complementation group A, lacking *XPA* DNA lesion recognition protein) and the equivalent repair-proficient cells (GM00637).

## MATERIALS AND METHODS

*Materials. trans*-α-Hydroxytamoxifen was synthesized according to the method described by Foster et al. (*29*) and was acetylated to prepare the ester *trans*-α-acetoxytamoxifen, using the published procedure (*20*). [$\gamma$-$^{32}$P]ATP (>185 TBq/mmol, >5000 Ci/mmol, 370 MBq/mL) was purchased from Amersham, (Buckinghamshire, U.K.). $T_4$ polynucleotide kinase (3′-phosphatase free) and calf spleen phosphodiesterase were from Roche (Lewes, East Sussex, U.K.), and all other chemicals were from Sigma (Poole, Dorset, U.K.) unless otherwise stated.

[1] Abbreviations: dG-$N^2$-tamoxifen, α-($N^2$-deoxyguanosinyl)tamoxifen.

*Shuttle Vector Plasmid, Bacterial Strain, and Cell Lines.* The plasmid pSP189 containing the *supF* gene (*28*, *30*) and *E. coli* strain MBM7070 were gifts from Dr. M. Seidman (NIA, NIH, Baltimore, MD). Human SV40 transformed xeroderma pigmentosum complementation group A fibroblasts (GM04429) and human SV40 transformed apparently normal cells (GM00637) were obtained from NIGMS Human Genetic Cell Repository (Camden, NJ). Cells were grown in Dulbecco's modified Eagle's medium (with Earle's salts) supplemented with 10% (GM04429) or 15% (GM00637) fetal calf serum (Life Technologies Ltd., Paisley, U.K.) at 37 °C in 5% $CO_2$ in air.

*Adduction of DNA with* α*-Acetoxytamoxifen.* Duplicate aliquots of pSP189 plasmid (200 μg) dissolved in 200 μL tris-EDTA buffer at pH 8.0 were incubated at 37 °C with various amounts of α-acetoxytamoxifen in 100 μL of ethanol to provide final concentrations of 10, 25, 50, and 100 μM. A control DNA incubation, to which only ethanol was added, was also carried out. After 18 h, the samples were extracted with 3 × 400 μL of water-saturated ethyl acetate to remove unreacted α-acetoxytamoxifen. The plasmid DNA was precipitated on addition of 3 M sodium acetate/ice-cold ethanol and then redissolved in 200 μL of sterile water. DNA adduct formation on the plasmid was detected and quantified using an established $^{32}$P-postlabeling HPLC assay for the analysis of tamoxifen adducts, as described previously (*31*). For each dose, aliquots of treated plasmid were $^{32}$P-postlabeled and analyzed in at least four separate experiments, and then the remaining plasmid was used in the *supF* assay to investigate the mutations induced by α-acetoxytamoxifen-derived DNA adducts.

*Transfection and Transformation.* Subconfluent cells were transfected (twice per plasmid dose) with α-acetoxytamoxifen-adducted plasmid (10 μg/9 cm culture plate) using the calcium phosphate precipitation technique (*32*) or using Fugene-6 transfection reagent (15 μL, Roche, Lewes, East Sussex, U.K.). The plasmid was recovered after a period of 48 h for XP cells and 60 h for normal cells, using plasmid purification kits (Qiagen, Crawley, West Sussex, U.K.). The time differential was to allow for an equal number of cells per transfection plate. Recovered plasmid was digested with *Dpn*I restriction enzyme (New England Biolabs, Hitchin, U.K.) to remove any unreplicated plasmid DNA. Electrocompetent MBM7070 *E. coli* were then transformed with aliquots of recovered plasmid by electroporation, using Gene Pulser apparatus (2.5 kV, 200 Ω, 25 μF) (Biorad, Hercules, CA). Typically, 7 transformations were performed for each plasmid sample recovered from a single transfection plate. Transformants were plated onto LB agar plates containing ampicillin (100 μg/mL), which selects for those cells

Table 1: Mutation Frequency Induced by α-Acetoxytamoxifen Adducted Plasmid Replicated in GM00637 and GM04429 Cells

| treatment ($\mu$M) | GM00637 cells mutation frequency | | GM04429 cells mutation frequency | | tamoxifen adducts per 10$^6$ nucleotides (mean[c] ± SD) |
|---|---|---|---|---|---|
| | actual[a] | relative[b] | actual[a] | relative[b] | |
| solvent control[d] | 12.27 | 1.00 | 7.06 | 1.00 | 0 ± 0 |
| 10 | 14.47 | 1.18 | 10.86 | 1.54 | 50 ± 9 |
| 25 | 15.12 | 1.23 | 14.13 | 2.00 | 210 ± 68 |
| 50 | 15.24 | 1.24 | 22.14 | 3.14 | 340 ± 125 |
| 100 | 58.99 | 4.81 | 121.21 | 17.17 | 800 ± 266 |

[a] Mutation frequency per 10$^4$ colonies. [b] Normalized to the solvent control and shows a fold increase over the control. [c] Individual plasmid samples were $^{32}$P-postlabeled between 4 and 9 times. [d] Mutation frequency of plasmid dissolved in water was $0.4 \times 10^{-4}$ (GM00637 cells) and $1.5 \times 10^{-4}$ (GM04429 cells).

containing the plasmid, 5-bromo-4-chloro-3-indolyl-β-D-galactose (X-gal) (75 μg/mL) and isopropyl-β-D-thiogalactoside (IPTG) (25 μg/mL).

*Sequencing.* When grown on X-gal containing media, mutant colonies are white or pale blue, whereas wild-type colonies are dark blue. Plasmid was extracted from the white (or pale blue) mutant colonies using plasmid purification kits (Qiagen, Crawley, West Sussex, U.K.) and sequenced using the primer 5′-GGCGACACGGAAATGTTGAA-3′ (Protein and Nucleic Acid Chemistry Laboratory, Hodgkin Building, University of Leicester, U.K.). All sequenced colonies contained the *supF* promoter region, including those where the *supF* gene had been deleted, proving that there was no Apr plasmid contamination. The pSP189 shuttle vector was developed containing an 8 base "signature sequence" (*30*), giving $4^8$ (65 536) possible unique sequences (*33*). Any mutants with a duplicated "signature sequence" were therefore excluded from further analysis. Hotspots were assigned when the number of mutations observed was 4-fold or more greater than the number expected for a random Poisson distribution. Mutation spectra were compared using the Cariello Hyperg program, where $p \leq 0.05$ indicates a significant difference (*34*).

## RESULTS

*$^{32}$P-Postlabeling Analysis of pSP189 Plasmid Reacted with α-Acetoxytamoxifen.* $^{32}$P-Postlabeling coupled with HPLC analysis was used to identify and quantify the adducts formed by *in vitro* reaction of α-acetoxytamoxifen with the shuttle vector plasmid pSP189. The adduct profiles (not shown) were identical to those that we reported previously (*26*, *31*), with α-acetoxytamoxifen treatment primarily resulting in formation of the major dG-*N*$^2$-tamoxifen DNA adduct that is detected in liver tissue of tamoxifen-dosed rats. An additional minor peak eluting just prior to the main adduct is also observed, which corresponds to an *N*-demethylated derivative of the dG-*N*$^2$-tamoxifen adduct (*35*). The total plasmid adduct level increased with increasing α-acetoxytamoxifen dose and was in the range of approximately 0.5−8.0 adducts per plasmid (4952 base pairs) for doses of 10−100 μM, respectively (Table 1). The *supF* target gene sequence (85 base pairs) accounts for 1.7% of the plasmid; therefore, assuming adduct formation is random throughout the plasmid, the frequency of tamoxifen adduct formation actually within the *supF* region varies from ∼0.01−0.14 adducts per gene.

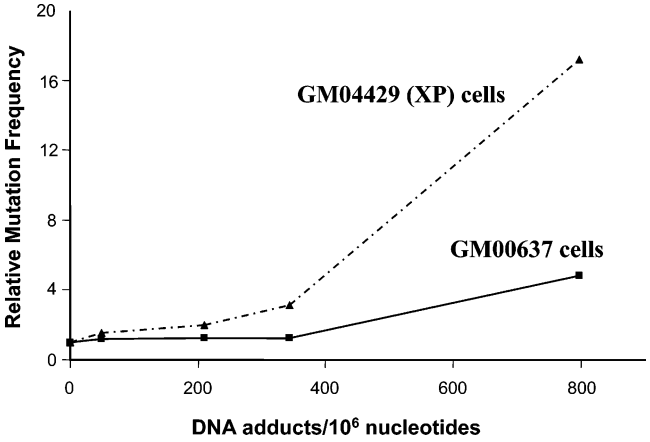


FIGURE 2: Graph illustrating the relationship between relative mutation frequency and level of DNA adducts for α-acetoxytamoxifen-treated pSP189 plasmid replicated in GM00637 or GM04429 (XP) cells. Relative mutation frequencies are compared to adduct levels rather than treatment dose, to account for any dosing inaccuracies.

*Mutation Frequency in the supF Gene.* Treatment of pSP189 plasmid with α-acetoxytamoxifen induced an increase in mutation frequency for all doses compared to the solvent control (ethanol) for both GM00637 and GM04429 cells, as illustrated in Table 1. The mutation frequency for control plasmid was higher than the spontaneous mutation frequency of plasmid in water, $12.27 \times 10^{-4}$ compared to $0.4 \times 10^{-4}$ for GM00637 cells and $7.06 \times 10^{-4}$ compared to $1.5 \times 10^{-4}$ for GM04429 cells. The mutation frequencies have been normalized to the solvent control (Table 1), to allow a better comparison of the relative mutagenicity of treated plasmid in the two cell lines. The lowest α-acetoxytamoxifen dose, 10 μM, produces an adduct level of 50/10$^6$ nucleotides in the treated plasmid and increased the mutation frequency by 1.18-fold relative to the control for GM00637 cells and 1.54-fold for XP cells. This elevated mutation frequency in XP cells was apparent up to a dose of 100 μM, which corresponds to a tamoxifen adduct level of 800/10$^6$ nucleotides (Figure 2). The relative increase in mutation frequency in XP cells over GM00637 cells reaches a maximum at this highest level of DNA damage, where replication of the plasmid in XP cells results in a 3.6-fold higher mutation frequency than in repair-competent GM00637 cells.

*Mutation Type Induced in the supF Gene.* Mutant colonies were isolated, and the *supF* gene was sequenced to determine the types and distribution of mutations induced by α-acetoxytamoxifen-derived DNA adducts in the two cell lines. Results from all doses were combined to present overall mutation spectra. In the repair-proficient GM00673 cells, there were insufficient numbers of mutant colonies induced by ethanol-treated control plasmid to produce a mutation spectrum. Three mutant colonies were sequenced revealing one with a single GC → CG base substitution and two colonies containing multiple base substitutions. Of these multiple mutations (13 sequence alterations), the GC → AT transition was the most likely mutation. More mutants were produced by the ethanol-treated control plasmid when replicated in GM04429 cells. A total of 40 were sequenced, and the majority were found to be single-base substitutions (40% of total mutations) or large deletions, defined as greater

Table 2: Types of Sequence Alterations in *supF* Gene of pSP189 Plasmids Treated with α-Acetoxytamoxifen Replicated in GM00637 and GM04429 Cells

| | number of plasmids with mutations (%) | |
|---|---|---|
| mutations | GM00637 | GM04429(XP) |
| base substitutions | 181 (90.5) | 180 (84) |
| single | 148 (74) | 150 (70) |
| tandem | 2 (1) | 2 (1) |
| multiple | 31 (15.5) | 28 (13) |
| frameshifts | 19 (9.5) | 34 (16) |
| single-base deletion | 4 (2) | 10 (4.5) |
| >2 bases deletion | 11 (5.5) | 23 (11) |
| single-base insertion | 4 (2) | 1 (0.5) |
| >2 bases insertion | 0 (0) | 0 (0) |
| total plasmids sequenced | 200 (100) | 214 (100) |

than 2 bases (40%), followed by multiple mutations (15%), along with a small amount of insertions (single and multiple bases, 2.5% each). The major single-base substitution was the GC → AT transition (44% of all single-base substitutions), with GC → TA and GC → CG transversions accounting for 19% each, AT → GC transitions for 13%, and AT → TA transversions for 6%.

As shown in Table 2, the majority of α-acetoxytamoxifen-induced mutations were in the form of single-base substitutions, corresponding to 74% of total mutations for GM00637 cells and 70% for GM04429 cells, followed by multiple mutations (15.5% for GM00637 cells and 13% for GM04429 cells). Tandem base substitutions account for 1% of all mutations for both cell lines, while single-base deletions are found in 2% of the GM00637 mutant colonies and 4.5% of the GM04429 mutant colonies. In comparison to the repair-deficient cells, where large deletions occurred with a frequency of 11%, they were less common in GM00637 cells, accounting for only 5.5% of all mutations observed.

Because tamoxifen preferentially reacts with guanine residues (*20*, *36*), the majority of base substitutions would be expected to occur at GC base pairs, as was found in both GM00637 and GM04429 cells, where there was a large preference for mutations at these sites (97 and 92%, respectively). Multiple base substitution mutations were also primarily induced at GC base pairs (73% in each case). Of the base substitutions occurring at GC base pairs, the predominant single-base substitution in the repair-proficient GM00637 cells is the GC → AT transition (42%), followed by GC → TA transversions (35.5%) and GC → CG transversions (19%), as shown in Figure 3. In these cells, multiple base substitutions at GC base pairs occur in the same order of preference, with GC → AT transitions accounting for 33%, GC → TA transversions accounting for 30%, and GC → CG transversions accounting for 10%. Multiple mutations also occurred with an increased frequency at AT base pairs, with 10% as AT → CG transversions and 12.5% as AT → GC transitions. In contrast, replication in the XP GM04429 cells results in a predominance of GC → TA transversions, comprising 62% of all single-base substitutions and 48% of all multiple substitutions. The profile of single and multiple substitutions differs in this cell line compared to the repair-competent cells, as illustrated in Figure 3. The proportion of GC → AT transitions and GC → CG transversions is similar, whether they are induced as single-base substitutions or as part of a multiple mutation, (22.5 versus 7% as single-base substitutions and 21.5 versus 4% as multiple base substitutions).

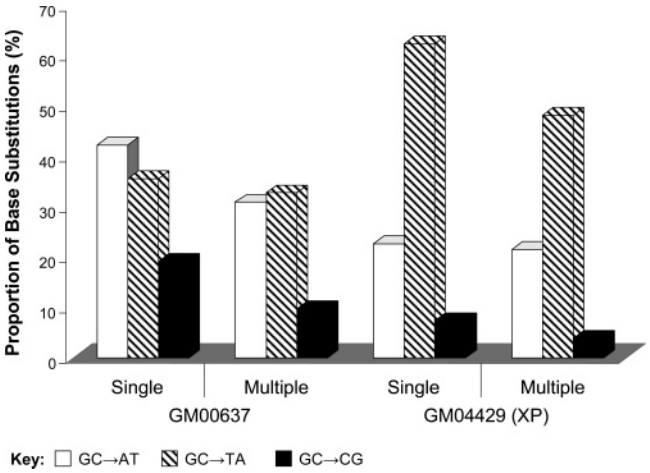


FIGURE 3: Bar chart illustrating the proportion of single (including tandem) and multiple base substitutions at GC base pairs induced by α-acetoxytamoxifen in GM00637 and GM04429 (XP) cells.

*Mutation Spectra in the supF Gene.* The distribution of single-base and tandem substitution mutations, along with multiple base substitutions, within the *supF* tRNA gene for control and α-acetoxytamoxifen-dosed plasmids (all doses combined) after replication in GM00637 or GM04429 cells, is shown in Figure 4. When these spectra are compared using the hyperg program (34), the pattern of mutations generated from α-acetoxytamoxifen-treated plasmids in the two cell lines is significantly different for single- and tandem base substitutions [$p$ (same) = 0.004]. However, when mutation spectra derived from multiple base substitutions are compared, there is no significant difference [$p$ (same) = 0.051].

Because there were only a low number of mutations induced by solvent control in GM00637 cells, there are no detectable sites of mutational hotspots. When all of the single and tandem substitutions arising from replication of α-acetoxytamoxifen-treated plasmid at every dose in GM00637 repair-proficient cells are combined, there are 10 visible hotspots, at positions 104, 108, 118, 123, 130, 133, 139, 144, 164, and 168. When the multiple mutations produced by all concentrations of α-acetoxytamoxifen are combined, seven hotspots are evident, at positions 108, 111, 129, 133, 149, 156, and 164. Hotspots common to mutation spectra from single- and tandem base substitutions and multiple base substitutions can be observed at positions 108, 133, and 164. All hotspots induced by treatment with α-acetoxytamoxifen are at positions of GC base pairs.

No hotspots were present in the mutation spectra induced by replication of control plasmid treated with ethanol only, after replication in GM04429 repair-deficient cells. When the single- and tandem base substitutions from all doses of α-acetoxytamoxifen-treated plasmids are combined, seven hotspots are apparent, at positions 122, 129, 133, 159, 160, 168, and 169 (Figure 4). Although a low number of multiple mutations were induced by α-acetoxytamoxifen-derived DNA adducts in the GM04429 cell line, in comparison to GM00637 cells, three hotspots at positions 156, 168, and 172 were observed. As with the repair-competent cells, all hotspots detected in spectra from adducted plasmid replicated in GM04429 cells were at GC base pairs. A comparison of single and tandem mutation spectra for both cell lines reveals

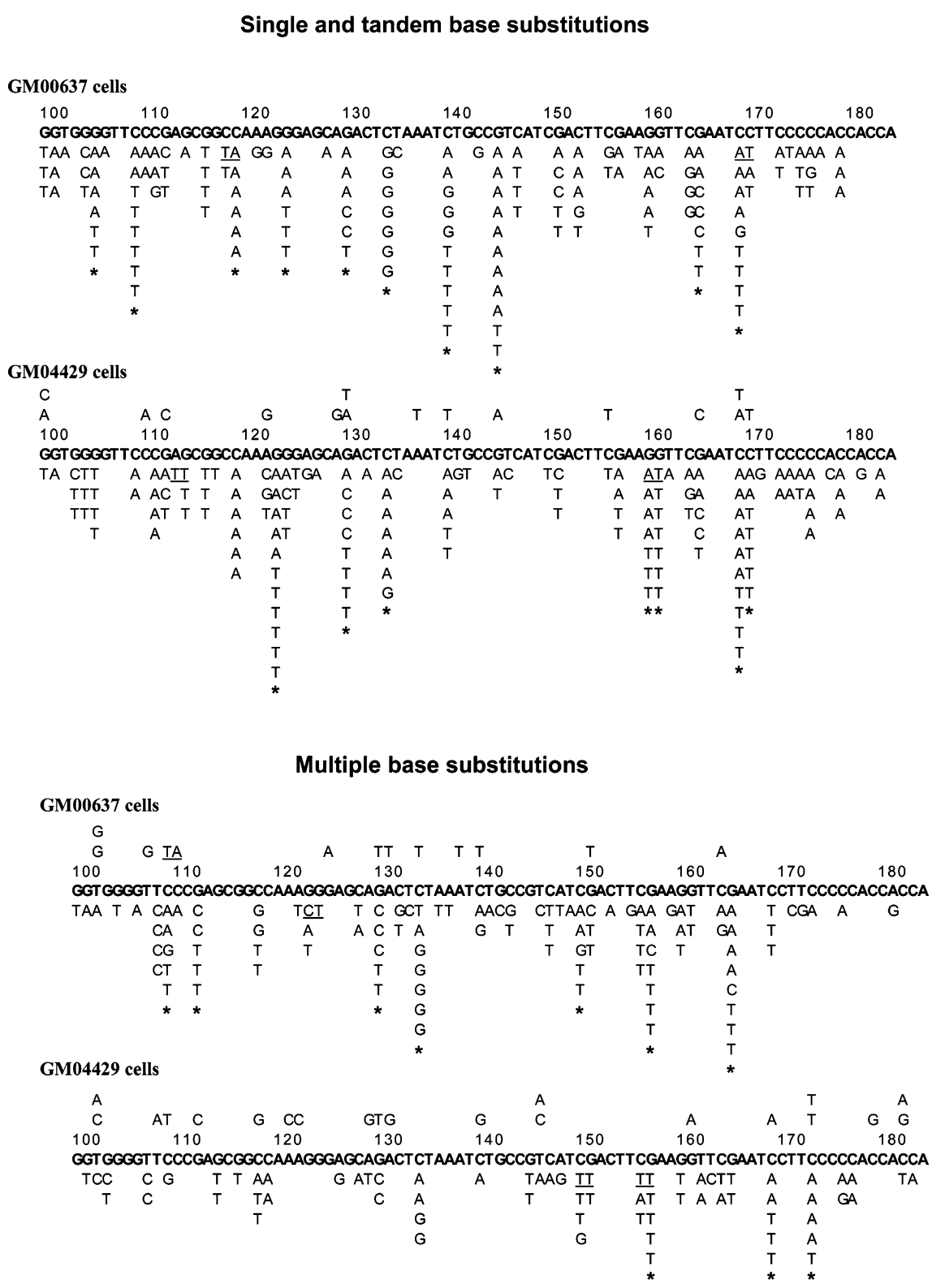


FIGURE 4: Mutation spectra induced by α-acetoxytamoxifen in GM00637 and GM04429 (XP) cells compiled using data from 10 to 100 μM doses. The 5′ to 3′ sequence of the transcribed strand of the wild-type *supF* gene is shown, with letters below the wild-type sequence indicating the position and type of point mutations induced by treatment with α-acetoxytamoxifen and letters above the wild-type sequence indicating the position and type of point mutations induced by solvent control. Mutation hotspots are denoted by an asterisk.

three common sites of mutation hotspots at positions 108, 133, and 164, while in the multiple mutation spectra, there is one common hotspot at position 168. The hotspot at position 133 is present in the single- and tandem base substitution mutation spectra for both cell lines along with the multiple spectrum for GM00637 cells.

## DISCUSSION

To evaluate the contribution of repair to the mutagenicity of tamoxifen, we have examined the pattern of mutagenesis induced in the *supF* gene by the reactive derivative, α-acetoxytamoxifen, when treated plasmid was replicated

in GM00637 repair-proficient and GM04429 XP repair-deficient cells. This compound produces the same major DNA adducts *in vitro* that are induced by tamoxifen metabolites in rat liver, primarily two trans diastereoisomers of dG-$N^2$-tamoxifen, with small amounts of the cis forms (*22*). The *supF* gene has been used as a target for mutagenesis by a wide range of mutagens, and consequently, there is a large database of information regarding the types and distribution of mutations induced in this sequence. This enables assessment of relative mutagenicity and the identification of characteristic spectra, as well as the potential to gain mechanistic information. In this study, treatment of plasmid with increasing concentrations of α-acetoxytamoxifen led to a dose-dependent increase in tamoxifen adduct levels, consistent with our previous findings (*26*). In both cell lines, the presence of this α-acetoxytamoxifen-induced DNA damage caused a dose-related increase in mutation frequency above background levels. The background mutation frequency in solvent-treated plasmid replicated in both GM00637 and GM04429 cells was slightly higher than we previously reported in an analogous study using human Ad293 kidney cells (*26*). Because the same batch of treated plasmid was transfected into all three cell lines, this implies differences in cell type are responsible for the higher number of mutations. Published data have also shown a marked variability in both background mutation frequency and distribution in different cell lines, tissues, and species (reviewed in ref *37*).

It is worth noting that in this study the levels of damage induced were relatively low, in the region of 0.5−8.0 adducts per plasmid. When Levy et al. (*38*) treated pSP189 plasmid with aflatoxin $B_1$-8,9-epoxide, generating 6−22 adducts per plasmid, the damaged plasmids gave mutation frequencies in the ranges of $6-26 \times 10^{-4}$ and $3-8.3 \times 10^{-4}$, when replicated in XP12BE (GM04429) and GM00637 cells, respectively. The highest mutation frequencies observed in our experiments were between 5- and 7-fold greater ($121 \times 10^{-4}$ and $59 \times 10^{-4}$, for XP and normal cells, respectively) but at much lower doses, suggesting that the dG-$N^2$-tamoxifen−DNA adduct is more mutagenic than the aflatoxin lesions. Replication of pZ189 plasmid treated with the chemotherapeutic agent *cis*-diamminedichloroplatinum(II) in GM00637 cells resulted in a mutation frequency of $8.2 \times 10^{-4}$ for a damage level of 2.5 adducts per plasmid, while at the same damage level in XP12BE cells, the mutation frequency was $350 \times 10^{-4}$ (*39*).

Importantly, while the observed mutation frequencies induced by each concentration of α-acetoxytamoxifen are similar in the two cell lines, when normalized to the respective controls, the higher mutation frequency in the XP cells indicates a reduced ability to repair the dG-$N^2$-tamoxifen−DNA adducts. Mutation frequencies for GM04429 (XP) cells are between 1.3- and 3.6-fold greater than the equivalent GM00637 mutation frequencies. This suggests that nucleotide excision repair plays a significant role in removal of these particular tamoxifen DNA adducts in this system (Figure 2). This is comparable to data from analogous studies with aflatoxin and UV, both of which caused an elevated mutation frequency in repair-deficient cells (*38*, *40*). Our findings are also consistent with the report that individual isomers of dG-$N^2$-tamoxifen adducts are excised when incubated with a reconstituted human nucleotide excision repair system, although with only poor to moderate efficiency (*41*). The rate of removal was dependent on the structure, with one of the cis isomers, a minor product of the reaction of trans-α-acetoxytamoxifen with DNA, being repaired most efficiently.

In both cell lines, mutations are preferentially targeted at GC base pairs; this is due to the fact that α-acetoxytamoxifen predominantly reacts with guanine. Interestingly, in XP cells, a greater proportion of single and tandem mutations (8%) are found at AT base pairs compared with normal cells (3%). α-Acetoxytamoxifen has been shown to bind to the amino group of adenine, when incubated *in vitro* with DNA (*42*), but this is a minor product of the reaction and nothing is known about the repair or mutagenic potential of this adduct or if it is formed *in vivo*. Efficient removal of these adducts by NER might explain the increased incidence of mutations observed at AT sites in the repair-deficient cells compared to the normal cells.

In human Ad293 kidney cells, we have previously shown that the most common mutations induced in the *supF* gene of α-acetoxytamoxifen-modified plasmid are GC → TA transversions (*26*). This is consistent with the major mutation in the *lacI* gene of transgenic rats administered tamoxifen (*25*) and also that induced in cellular systems or *in vitro* studies in which replicative polymerases primarily misincorporated dA opposite dG-$N^2$-tamoxifen adducts (*23*, *43*). In this study, however, the repair-proficient GM00637 cells show a slight preference for the induction of GC → AT transitions, whereas in XP cells GC → TA transversions are the major single-base substitution. Of the base substitutions induced as part of a multiple mutation, the predominant mutation in XP cells is also the GC → TA transversion, while in GM00637 cells, GC → TA transversions are slightly more prevalent than GC → AT transitions. The frequency of GC → CG transversions also varies between the cell types, being more common in GM00637 cells compared to XP and as single-base substitution rather than a multiple base substitution. Another difference between normal and XP cells is the increased level of deletions of both single and multiple bases. In primer extension reactions using templates containing individual isomers of dG-$N^2$-tamoxifen, the human DNA polymerase $\beta$ has been shown to frequently give products with 5 bases deleted, particularly opposite cis forms of the adduct (*43*). The trans forms promoted smaller amounts of 5 base deletions, and all four isomers of dG-$N^2$-tamoxifen induced 1 and 2 base deletions. Therefore, less efficient removal of these adducts in XP cells would be predicted to result in a higher proportion of deletions compared to amounts induced in normal cells, as was found in the present study.

In the single- and tandem base substitution mutation spectra for the two cell lines, there were several mutation hotspots in common (Figure 4), at positions 108, 133, and 164. In the multiple mutation spectra, there is also a common hotspot at position 168. In addition, the hotspot induced at position 133 in the single- and tandem base substitution spectra for GM00637 cells is also observed in the multiple spectrum for GM00637 cells, along with the single (and tandem) spectrum for XP cells. It has previously been shown that reduced DNA repair capacity can influence the distribution of mutations within a gene, when plasmids containing

aflatoxin or UV-induced lesions are replicated in repair-proficient and -deficient cell lines (*38*, *40*).

The proportion of multiple mutations does not seem to differ greatly between the cell lines [16% in GM00637 cells and 13% in GM04429 (XP) cells]. It has been suggested that multiple mutations arise through a different mechanism to single-point mutations (*44*) and that, during nucleotide excision repair, gap-filling error prone DNA polymerases introduce incorrect bases at sites of lesion excision and are then subject to further misincorporation errors, leading to the accumulation of multiple mutations on the same strand. This mechanism has been proposed to explain the 10−15-fold higher number of multiple mutations observed in NER-proficient cells compared to XP cells, when UV-damaged plasmids undergo replication (*45*). An effect of this magnitude was not observed in this study but was similar to the increase reported for aflatoxin in this system (*38*). This may suggest that tamoxifen−DNA adducts are repaired less efficiently than UV-induced damage, which is consistent with the fact that T(6−4′)T photoproducts are repaired much more efficiently than dG-$N^2$-tamoxifen adducts using a reconstituted human excision nuclease (*41*). It is also possible, however, that multiple mutations may arise as a consequence of several damaged sites on a single plasmid, but considering the low level of adducts generated in this study, it would seem somewhat unlikely. Even at the highest dose of 100 $\mu$M, where there are 8.0 adducts per plasmid, this would only equate to 0.14 adducts per *supF* gene (i.e., ~1 *supF* gene in 7 will contain an adduct), assuming adduct formation is random throughout the plasmid.

Recently, it has been reported that mutations induced in the *Hprt* gene by α-sulfate-tamoxifen in Chinese hamster cells were nearly all observed in the nontranscribed strand, indicating preferential removal of tamoxifen adducts from the transcribed strand (*46*). This is in contrast to findings obtained in the *LacI* and *cII* genes of transgenic animals administered tamoxifen, in which there was no difference in the frequency of mutations on either strand (*24*, *25*, *47*). It has been proposed this is because *Hprt* is a transcriptionally active housekeeping gene, subject to transcription coupled repair, whereas the transgenes are artificially incorporated. In the present study, of the mutations occurring at GC base pairs, there did not appear to be a significant difference in the proportion of mutations occurring on each strand. Given previous results, this lack of strand bias in the present study is most likely due to the fact that the *supF* gene is in an artificial environment.

In conclusion, this study illustrates that the major tamoxifen DNA adducts detected in the liver of rats and also endometrial tissue of treated women are substrates for the human nucleotide excision repair system, although they do not seem to be removed with high efficiency. These adducts are highly mutagenic in human cells, with the types and position of mutations induced being cell-specific. Therefore, if not excised, dG-$N^2$-tamoxifen adducts may potentially contribute to the initiation of endometrial carcinogenesis in women. Studies aimed at identifying whether characteristic mutational spectra are induced in endometrial tumors of women treated with tamoxifen and whether mutation hotspots correlate with preferential sites for tamoxifen-induced damage will aid in assessing the role of tamoxifen DNA adducts in endometrial carcinogenesis (*48*).

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