Site-Specific Tamoxifen−DNA Adduct Formation: Lack of Correlation with Mutational Ability in *Escherichia coli*[†,‡]

Damon A. Lowes, Karen Brown, Robert T. Heydon, Elizabeth A. Martin, and Timothy W. Gant*

Medical Research Council Toxicology Unit, University of Leicester, Leicester, LE1 9HN, United Kingdom

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ABSTRACT: We have mapped sites of tamoxifen adduct formation, in the *lacI* gene using the polymerase STOP assay, following reaction in vitro with α-acetoxytamoxifen and horseradish peroxidase (HRP)/H_2O_2 activated 4-hydroxytamoxifen. For both compounds, most adduct formation occurred on guanines. However, one adenine, within a run of guanines, generated a strong polymerase STOP site with activated 4-hydroxytamoxifen, and a weaker STOP site with α-acetoxytamoxifen at the same location. In *Escherichia coli* the *lac I* gene reacted with 4-hydroxytamoxifen was more likely to be mutated (2 orders of magnitude) than when reacted with α-acetoxytamoxifen, despite the greater DNA adduct formation by α-acetoxytamoxifen. This correlates with the greater predicted ability of activated 4-hydroxytamoxifen adducts to disrupt DNA structure than α-acetoxytamoxifen adducts. For *lac I* reacted with activated 4-hydroxytamoxifen, a hot spot of base mutation was located in the region of the only adenosine adduct. No mutational hot spots were observed with α-acetoxytamoxifen. Our data clearly shows a lack of correlation between gross adduct number, as assayed by ^{32}P-postlabeling and mutagenic potential. These data indicate the importance of minor adduct formation in mutagenic potential and further that conclusions regarding the mutagenicity of a chemical may not be reliably derived from the gross determination of adduct formation.

The nonsteroidal anti-estrogen, tamoxifen, (Z-1-[4-(dimethylaminoethoxy)phenyl]1,2-diphenyl-1-butene), is widely used in the treatment of breast cancer and is currently undergoing clinical trials as a chemopreventive agent in women thought to be at a high risk of developing this disease (*1*).

However, tamoxifen is hepatocarcinogenic in rats following short- (*2*) and long-term (*3*−*6*) dosing and causes endometrial cancer in women (*7*). Following tamoxifen treatment, large numbers of DNA adducts are formed in the livers of rats that accumulate in a time (*6*) and dose dependent manner (*8*).

Tamoxifen requires metabolic activation to an electrophilic species before it can bind to DNA, and at least two distinct metabolic pathways lead to adduct formation. The first is via α-hydroxylation (*9*, *10*) and the second via 4-hydroxylation leading to the formation of a reactive quinone methide (*11*, *12*). There is strong evidence that α-hydroxytamoxifen is the major proximate carcinogen because it produces a similar pattern of ^{32}P-postlabeled DNA adducts to tamoxifen in rat hepatocytes treated in vitro (*13*) and in vivo (*14*).

It has been proposed that α-hydroxytamoxifen is activated to a reactive ester by conjugation, probably sulfation, and subsequent loss of this sulfate group generates an allylic carbocation that reacts with nucleophilic sites on DNA (*15*). The major products of the reaction between DNA and model esters of tamoxifen, α-acetoxytamoxifen and α-sulfate tamoxifen in vitro, are deoxyguanosine adducts in which the α-carbon of tamoxifen is covalently linked to the exocyclic amino group of deoxyguanosine (dG-N^2-tamoxifen)(Figure 1) (*10*, *16*, *17*). Since the carbocation arising from these esters readily undergoes rotation of the central double bond, both cis and trans forms of this adduct are produced. In addition, as the α-carbon of tamoxifen is chiral each geometric isomer exists as two diastereoisomers. These adducts have been shown to co-elute with the major adduct peaks formed in the livers of rats administered tamoxifen (*14*, *16*). A minor tamoxifen deoxyadenosine adduct has also been identified in which linkage is through the amino group (*17*).

4-Hydroxytamoxifen can be oxidized chemically, or by microsomal and peroxidase systems, to form a reactive intermediate capable of producing DNA adducts (*11*, *12*, *18*). Adducts arising from this activation pathway are more polar than those described above (*14*) and are probably the 4-hydroxylated forms of the dG-N^2-tamoxifen adducts (dG-N^2-4-hydroxytamoxifen) (Figure 1) (*11*, *19*).

Tamoxifen is inactive in a number of standard short-term tests used for the detection of chemical mutagens (*20*) but is mutagenic in the livers of lambda/*lacI* transgenic rats (*21*). In this system, tamoxifen causes a 3-fold increase in *lacI* gene mutation frequency in liver DNA. The mutations occur mainly at CpG sites, (*22*) but with no one site appearing as a hot spot, that is, more frequently mutated than similar sites elsewhere. Although the majority of the mutations occurred at CpG sites, a significant number are also observed at TpA sites.

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* To whom correspondence should be addressed. Medical Research Council Toxicology Unit, Hodgkin Building, University of Leicester, Lancaster Road, Leicester, LE1 9HN United Kingdom. Telephone: +44 (0) 116 252−5579. Fax: +44 (0) 116 252-5616. E-mail: twg1@le.ac.uk.

[1] Abbreviations: BSA, Bovine Serum Albumin; EDTA, Ethylenediaminetetraacetic acid; HRP, Horseradish Peroxidase; HEPES,(*N*-[2-Hydroxyethyl]piperazine-*N*′-[4-butanesulphonic acid]); STOP, site of adducted base inhibiting DNA polymerase progression.

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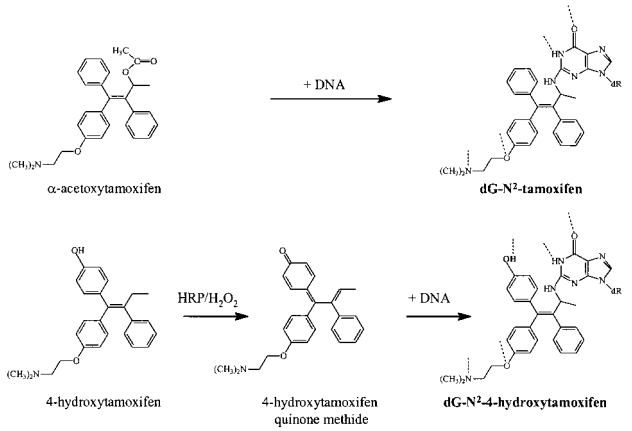

FIGURE 1: Pathways of in vitro tamoxifen-guanine adduct formation. The structures of the adducts formed by the α-acetoxytamoxifen and HRP/H2O2-activated 4-hydroxytamoxifen on guanine are shown. Dashed lines indicate potential sites for hydrogen bonding.

The ability of an adduct to inhibit progression of DNA polymerase is adduct and polymerase specific. Maximal mutation frequency occurs when a polymerase is able to synthesize past an adduct (translesional DNA synthesis) (*23*−*26*). For example, when templates containing dA-N^6-benzo-[a]pyrene-7,8-dihydrodiol-9,10-epoxide adducts are transcribed in vitro with different polymerases, the *exo*-Klenow fragment of *E. coli* DNA polymerase causes the most mutations during DNA synthesis due to it's ability to read past the adduct but insert the wrong base. Conversely, T4 DNA polymerase synthesis is completely inhibited by this adduct and generates no mutations in the daughter strand (*27*). Therefore, in vivo unrepaired adducts that inhibit DNA polymerase progression are likely to be cytotoxic, whereas those that permit translesional DNA synthesis are more likely to be mutagenic, dependent on their miscoding potential. dG-N^2-Tamoxifen adducts have miscoding potential in vitro with the mammalian pol α and β DNA polymerases, but not polymerase δ (*28*). These mutations occur during translesional DNA synthesis that results in either wrong base insertion, or small deletions, with the exception of DNA polymerase δ, which inserts the correct base opposite the lesion. In the same system with dA-N^6-methoxyestratrienyl adducts, a different spectrum of DNA polymerase activity is seen. In this case, the dA-N^6-methoxyestratrienyl adduct blocks the progression of DNA polymerase δ, but DNA polymerase α and β are able to synthesize past the lesion resulting, in most instances, with correct incorporation of dTTP but additionally some incorrect base insertions, and small deletions (*23*).

In this paper, we have used the polymerase STOP assay to determine the sites of adduct formation on the *lacI* gene following its reaction with α-acetoxytamoxifen and HRP/H_2O_2 activated 4-hydroxytamoxifen. The ability of these adducts to cause mutations in *E. coli* are evaluated, and these data compared with both gross and site-specific adduct formation.

EXPERIMENTAL PROCEDURES

Materials and Methods. The pLIZ plasmid (Stratagene) is the lambda shuttle vector, containing the entire *E. coli lacI* gene as a target for mutagenesis. 4-Hydroxytamoxifen was obtained from Sigma (Poole,UK), and α-acetoxytamoxifen was synthesized from α-hydroxytamoxifen, according to the method of Osborne *et al.* (*16*). All other chemicals were obtained from Sigma (Poole, Dorset, UK) unless otherwise stated.

Adduct Formation on pLIZ with Activated 4-Hydroxytamoxifen. 100 μg of *pLIZ* plasmid DNA was dissolved in 340 μl of buffer containing 50 mM HEPES−NaOH (pH6.2), 0.2mM EDTA, 0.03% TWEEN 20, and 500 μM H_2O_2. 4-Hydroxytamoxifen was added to final concentrations of 10, 25, 50, 100, 160, and 250 μM and the reaction preincubated at 37 °C for 5 min. The reactive 4-hydroxytamoxifen quinone methide was generated by the addition of 10 μl of horseradish peroxidase (HRP) (5 mg/ml) to the reaction, followed by incubation at 37 °C for 30 min. After 30 min, the reactions were stopped by the addition of 800 μl ice-cold chloroform. Unreacted 4-hydroxytamoxifen/4-hydroxytamoxifen quinone was removed by extraction with water-saturated ethyl acetate (3 × 200 μl), and the DNA precipitated from the aqueous phase using sodium acetate/ethanol. Control reactions were performed by incubating the pLIZ plasmid without 4-hydroxytamoxifen, in the presence or absence of the HRP/H_2O_2 activating system.

Adduct Formation on pLIZ with α-Acetoxytamoxifen. 100 μg of *pLIZ* plasmid DNA was dissolved in 100 μl of buffer containing 10 μM EDTA, 0.15 M NaCl, and 15 mM SSC pH8.8. α-Acetoxytamoxifen was added in ethanol to final concentrations of 10, 25, 50, 100, 160, and 250 μM, and the reactions were incubated at 37 °C overnight. Controls contained ethanol alone. The samples were extracted three times with 100 μl ethyl acetate to remove unreacted α-acetoxytamoxifen and the DNA precipitated as above.

^{32}P-Postlabeling Analysis of Plasmid DNA. Plasmid DNA was subjected to ^{32}P-postlabeling as described previously (*2*). Briefly, DNA was digested using micrococcal nuclease and calf spleen phosphodiesterase (Boehringer-Mannheim). The digestion mix was further digested with nuclease P1 to convert unadducted nucleotides to nucleosides. Adducted nucleotides were subsequently ^{32}P-radiolabeled by 5′ phosphorylation using [γ-^{32}P]ATP (>5000 Ci/mmol, 10 mCi/mL; Amersham International) and 3′-phosphatase-free T4 polynucleotide kinase (Boehringer-Mannheim). ^{32}P-Labeled adducts were separated by HPLC with on-line radiochemical detection (*14*). For each dose (10, 25, and 50 μM), there were three DNA samples that were each analyzed twice (for each sample, $n = 6$). Mean levels of adducts ± SD are expressed as relative adduct labeling, RAL x 10^8.

DNA Polymerase Arrest (STOP) Assay. The STOP assays were carried out using primer 5′-GTACCCGACACCATC-GAATG-3′ that corresponded to bases from −82 to −62 where the adenine of the *lac I* translation initiation codon is +1. The transcribed (noncoding) strand of the *lacI* gene acts as the template using this primer. The primer was ^{32}P-end-labeled using [γ-^{32}P]-ATP (Amersham) and T4 polynucleotide kinase (Promega). Primer extension was carried out in a total volume of 20 μl containing 3 μg adducted or unadducted *pLIZ* plasmid DNA, 67mM Tris-HCl (pH 8.8), 16 mM $(NH_4)_2SO_4$, 6.7 mM $MgCl_2$, 6.7 μM EDTA, 167 μg/mL BSA, 10 mM β-mercaptoethanol buffer, 18 pmoles labeled primer, and 250 μM of each dNTP. The DNA was denatured by heating to 95 °C for 2 min, and the primer annealed during subsequent cooling to 4 °C. Extension was initiated by the addition of 4 units of T4 DNA polymerase.

Elongation was allowed to proceed for 30 min at 37 °C before the addition of 5 μl stop solution (10 mM NaOH, 95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanole). DNA was denatured by heating to 95 °C followed by cooling on ice. The DNA fragments were resolved on a 0.4 mm by 60 cm 6% polyacrylamide gel containing 8 M urea using a tris-taurine-EDTA buffer system (Amersham). The gel was run at 60 W that maintained a temperature of 50 °C. Running time was approximately 2.5 h. Gels were fixed in a 10% methanol, glacial acetic acid solution for 15 min and dried on Whatman 3MM paper. Gels were visualized using a Molecular Dynamics Phosphorimager (Sunnyvale, CA). The DNA sequencing ladder was produced using the Promega fmol DNA sequencing system. Chemical stability of the adducts to heating to 95 °C for 5 min was assessed by ^{32}P-postlabeling of DNA before and after heating to 95 °C for 5 min.

Transformation of SCS8 E. coli with Tamoxifen Adducted pLIZ. Competent SCS8 *E. coli* were produced from a SCS8 stock obtained from Stratagene using standard procedures and transformed with 25 ng of unadducted or adducted pLIZ plasmid using a 42 °C heat shock procedure. Colonies were grown on agar containing 100 μg/mL ampicillin, 80 μg/mL X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside). Blue mutant colonies were counted and expressed as a percentage of nonmutant white colonies. Mutant colonies were picked, grown, and the recovered pLIZ plasmid sequenced for *LacI* gene mutations. For each transformation, $30-60 \times 10^3$ colonies were screened.

Automated DNA Sequencing. Control and mutant *LacI* gene were cycle sequenced using the ABI prism big dye terminator cycle sequencing system with AmpliTaq DNA polymerase using the same primer as used for the polymerase stop assay. Reaction products were analyzed on a Perkin-Elmer Applied Biosystems 377 DNA sequencer.

RESULTS

^{32}P−Postlabeling. DNA reacted with α-acetoxytamoxifen or activated 4-hydroxytamoxifen was analyzed by ^{32}P-postlabeling prior to use in the subsequent assays. Distinctly different HPLC profiles were exhibited following reaction with either α-acetoxytamoxifen or activated 4-hydroxytamoxifen. The α-acetoxytamoxifen reacted samples gave 2 major and several minor peaks. HRP/H_2O_2 activated 4-hydroxytamoxifen gave one major peak, more polar than those of the major peaks from α-acetoxytamoxifen (Figure 2). A dose dependent increase in DNA adduct levels was observed for α-acetoxytamoxifen, with levels increasing from 6571 ± 2432 adducts/10^8 nucleotides at 10 μM, to 26838 ± 4796 adducts/10^8 nucleotides at 50 μM. Unlike α-acetoxytamoxifen, activated 4-hydroxytamoxifen adduct levels did not increase over this dose range and were approximately 3601 ± 108 adducts/10^8 nucleotides at all three dose levels (Figure 2).

Analysis of DNA Adduct Formation by STOP Assay. The STOP assay was optimized using a number of DNA and RNA polymerases, some of which were thermostable and of eukaryotic and prokaryotic origin (data not shown). The criteria on which the optimization was based was of a clear indication of polymerase STOP sites in the adducted DNA. The majority of polymerases were either of low sensitivity or insensitive to tamoxifen adducts, including both the exo$^-$ and exo$^+$ Klenow fragments of *E. coli* DNA polymerase. Optimal conditions were obtained using T4 DNA polymerase with a single read through of the DNA using an end labeled primer. These data are in common with previous results showing that T4 DNA polymerase is more sensitive to inhibition of DNA synthesis progression by adducts (*26*, *27*). The chemical stability of the DNA adducts from both 4-hydroxytamoxifen and α-acetoxytamoxifen to heating to 95 °C for 5 min was assessed by ^{32}P-postlabeling. No differences were found in the gross adduct levels in the heated, verses not, samples indicating that this heating step in the STOP assay protocol was unlikely to have any effect on the experimental data obtained (data not shown).

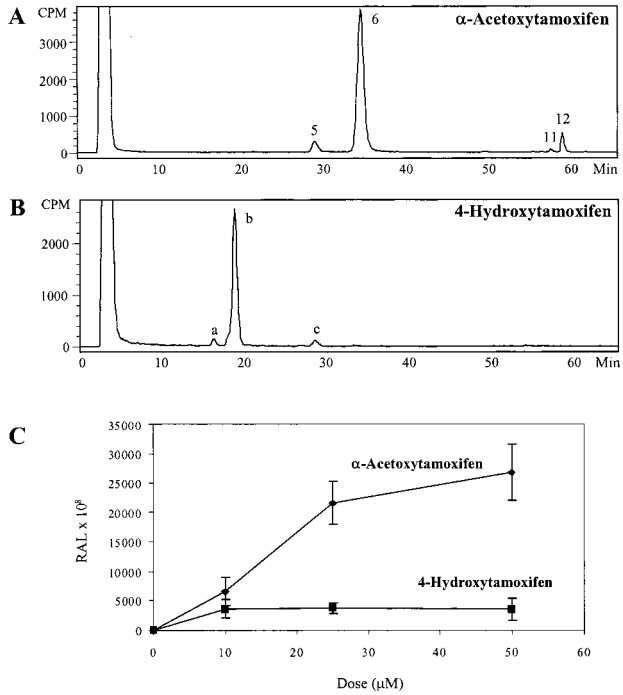

FIGURE 2: Comparison of HPLC separation of ^{32}P-postlabeled DNA adducts formed by α-acetoxytamoxifen and activated 4-hydroxytamoxifen. Panel A, pLIZ DNA reacted with 10μM α-acetoxytamoxifen (0.5 μg DNA). Panel B, pLIZ DNA reacted with 10 μM activated 4-hydroxytamoxifen (0.5 μg DNA). Numbering system for adduct identification can be found in ref *14*. Panel C, Relationship between adduct levels and concentration of activated tamoxifen metabolite 0−50 μM, correlating to the concentrations used in the mutation study, mean (n = 6) and SD shown.

A similar spectrum of DNA adducts was obtained from the *lacI* gene adducted with activated 4-hydroxytamoxifen and α-acetoxytamoxifen using T4 DNA polymerase in the STOP assay (Figure 3). It is not known for these tamoxifen adducts and T4 DNA polymerase, if the polymerase stops on the adducted base or one base prior or post to the adduct. All three of these scenarios are possible. However, the balance of probabilities suggests that T4 polymerase stopped on the tamoxifen-adducted base. This is evidenced by the data fitting which, if T4 is assumed to halt on the adducted base, identifies the STOP sites as guanines and one adenine (Figure 3). Published data indicate that tamoxifen forms most adducts on guanines with adenosine as a minor adduct site (*11*, *16*−*18*). If the data is fitted with the T4 halting either before or after the adduct, then the spectrum of base adduct formation does not correlate with the spectrum that would be expected from the theoretical chemistry and published data.

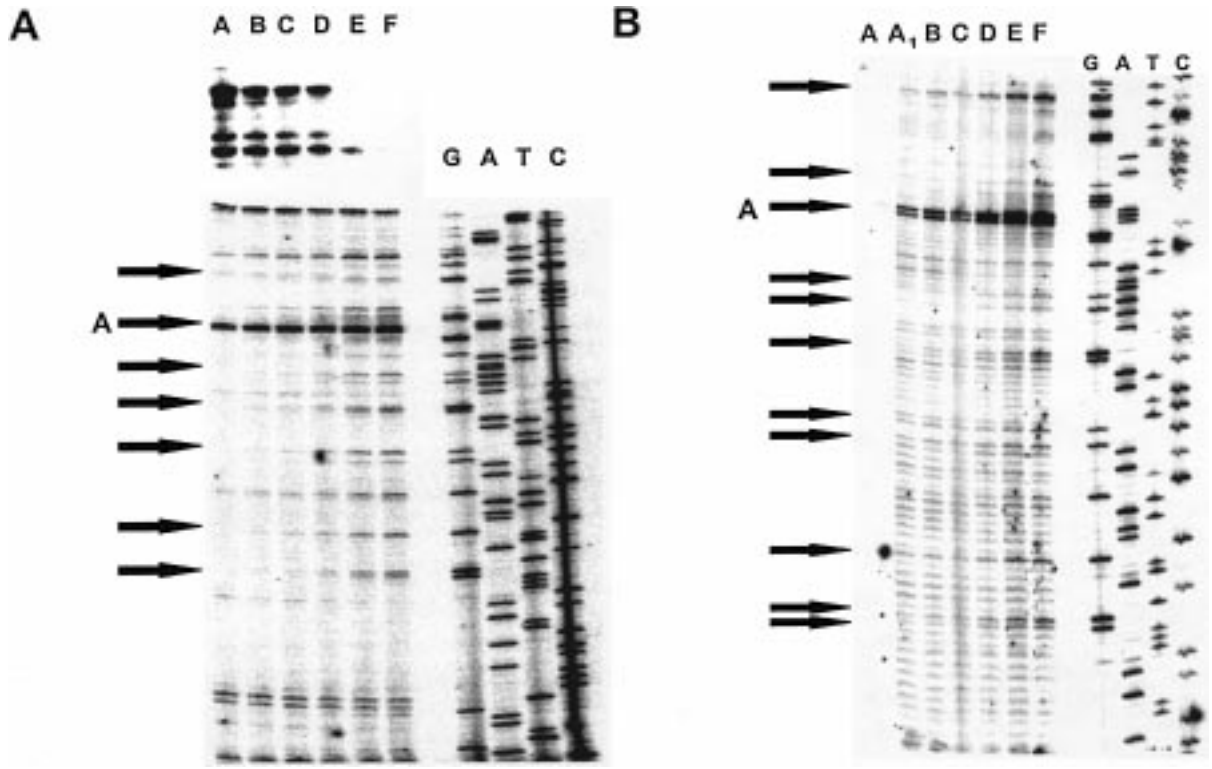

FIGURE 3: Analysis of adduct sites on lambda pLIZ after adduct formation in vitro with α-acetoxytamoxifen or activated 4-hydroxytamoxifen. STOP assay pattern generated from the in vitro adducted *lacI* gene with α-acetoxytamoxifen (Panel A) and activated 4-hydroxytamoxifen (Panel B). The primer was situated between bases −82 to −62 bp upstream of the *lacI* gene translation initiation site and corresponded to the coding (sense) sequence. T4 DNA polymerase was used to synthesize the new DNA. Lane A Control (No peroxidase or no α-acetoxytamoxifen), A_1 activation system only (no 4-hydroxytamoxifen), lanes B to F, 25, 50, 100, 160, 250 μM compound, respectively. The STOP sites correspond to sites of inhibition of polymerase on the antisense strand, which was being read. The labeling of the sequencing ladder reflects this with the bases being reversed to give the sequence of the same strand. The upper panel of A shows the decreasing amount of full length product formed as the number of adducts increased. No full length product was generated with DNA adducted with activated 4-hydroxytamoxifen. Arrows indicate representative polymerase STOP sites with the adenine adduct marked. One experiment typical of three is shown.

Adduction on adenosine was only detected at one site by the polymerase STOP assay. This site was located between two runs of three guanines (Figure 3A and Figure 5). This region was also a natural pause site, which could, by virtue of adducts on the guanine, be artifactual. However, for activated 4-hydroxytamoxifen, this was also a very prominent mutational hot spot (Figure 5B and Table 1). Mutations were not observed here in either the untreated or HRP/H_2O_2–-treated plasmids (Table 1). The same lesion was observed for α-acetoxytamoxifen, but in this case, the STOP generated was less pronounced when compared with the natural pause site that shows up strongly in the control. In addition, for α-acetoxytamoxifen, this was not a mutation hot spot.

Pattern of Mutagenesis in E. coli. The adduct mutagenic potential was investigated by transforming SCS-8 *E. coli* with the adducted plasmids. This strain is mutant for *lacI* and the alpha complementation fragment of β-galactosidase, and forms blue colonies with transformed plasmids that are mutant for *lacI*. These can be counted and expressed as a percentage of the white (wild type) colonies as a measure of mutation frequency. Plasmids treated with increasing concentrations (0−30 μM) of either activated 4-hydroxytamoxifen or α-acetoxytamoxifen were used for transformation of SCS-8 *E. coli*. For the activated 4-hydroxytamoxifen plasmid, a control reaction containing HRP/H_2O_2 was included. The HRP/H_2O_2 treatment caused a significant number of mutations, and, therefore, the mutagenicity of the 4-hydroxytamoxifen adducts are assessed by comparison with this control. Adduction with activated 4-hydroxytamoxifen, but not α-acetoxytamoxifen, caused a dose dependent decrease in transformation ability. This limited the maximum concentration of activated 4-hydroxytamoxifen that could be utilized to 30 μM in this assay. DNA adducts formed from reaction with activated 4-hydroxytamoxifen and α-acetoxytamoxifen were mutagenic in this strain of *E. coli* (Figure 4). Both 4-hydroxytamoxifen and α-acetoxytamoxifen were significantly mutagenic at 1 μM. The mutagenicity of 4-hydroxytamoxifen increased to 20 μM before decreasing in contrast to the α-acetoxytamoxifen adducted plasmid whose mutagenicity decreased from a peak at 1 μM. The mutation frequency we observed with α-acetoxytamoxifen was about 2 orders of magnitude less than that for activated 4-hydroxytamoxifen (Figure 4).

Mutagenic Spectrum of the Tamoxifen Adducted lacI Gene. The mutagenic spectrum of the *lacI* gene determined from plasmids extracted from "blue" mutant SCS-8 *E. coli* for both activated 4-hydroxytamoxifen and α-acetoxytamoxifen are shown in Figure 5. The mutational data for all the experiments are shown in Table 1. For activated 4-hydroxytamoxifen, the most frequently observed mutation was a G>C transversion. This was largely due to the contribution of the G>C transversion commonly observed at base 64. For all the observed G>C transversions, an adduct was located at the guanine. There was an obvious mutation hot spot around the sequence GGCAAAGGG (+58 to +66 bp)

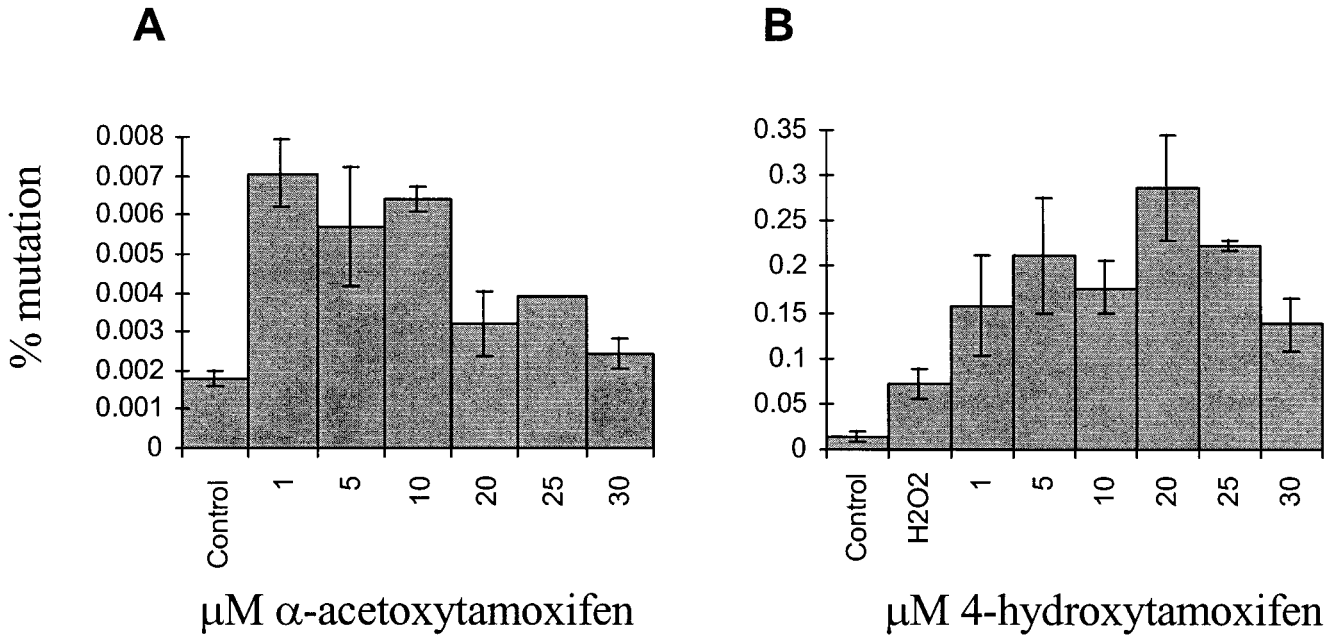

FIGURE 4: Frequency of mutation in SCS-8 *E. coli* of the in vitro adducted pLIZ plasmid containing the lacI gene. The pLIZ plasmid adducted in vitro with α-acetoxytamoxifen (panel A) or activated 4-hydroxytamoxifen (panel B) at the concentrations shown. This plasmid was then used to transform competent SCS-8 *E. coli*, which were selected for using ampicillin. The mutant colonies were detected by their ability to hydrolyze *β*-galactosidase. The number of mutant colonies are expressed as a percentage of the white (non mutant plasmid) colonies. The mean and SD of three experiments is shown.

with a strong STOP site corresponding to the first one or two adenines of this sequence. Here, there was a high frequency of transversion on the first guanine following the adenine run. For α-acetoxytamoxifen only a few mutations were observed, and there were no obvious hot spots. Additionally, here, the transversions were of G>T and T>A rather than the G>C transversions seen with activated 4-hydroxytamoxifen (Figure 4).

DISCUSSION

Tamoxifen forms significant DNA adduct levels in the livers of treated rats (6, 8). The major in vivo adducts formed in vitro by the reaction of DNA with α-acetoxytamoxifen or α-sulfate tamoxifen, having been identified as the 4 diastereoisomers of dG-N^2-tamoxifen (*10*, *16*, *17*, *29*). DNA reacted with HRP/H_2O_2-activated 4-hydroxytamoxifen forms different adducts, which are more polar and present at only low levels in the livers of tamoxifen treated rats (*14*). In common with these data, the adducts we obtained following reaction of the pLIZ plasmid with activated 4-hydroxytamoxifen and α-acetoxytamoxifen were different and could be separated by HPLC (Figure 2).

In rats transgenic for the *lacI* gene, administration of tamoxifen results in an increased mutation frequency of this gene (*21*). This assay system relies on the packaging of the phage LIZ plasmid from rat DNA and subsequent mutation detection in SCS-8 *E. coli*. Mutations detected by this system may arise from eukaryotic repair mechanisms in the rat. However, it is still possible to hypothesize that adducts may be transferred during transgene extraction, and the mutations occur during *E. coli* propagation. Tamoxifen adducts are very stable in vivo, with a half-life of several weeks (*2*), which gives some measure of support to this latter hypothesis. Though tamoxifen has been demonstrated to form adducts with guanine in vivo, the possible sequence specificity of adduct formation has not been investigated, and there has been no correlation with mutation frequency. In this study, we sought to examine the sequence specificity of adduct formation using two activated metabolites of tamoxifen and correlate this with the mutagenic spectrum generated in SCS-8 *E. coli*.

Our data with the T4 polymerase STOP assay shows that for both α-acetoxytamoxifen, and activated 4-hydroxytamoxifen, the major site of adduct formation was on guanine, with all observed guanines showing STOP sites. This correlates with previous studies that have also shown that guanine is the major site of tamoxifen adduct formation (*16*). We also observed a site of adduct formation on adenine. This adduct was located within a triplet of adenines located between two runs of guanines. An adenine adduct has been previously identified as a minor product following the reaction of DNA with α-acetoxytamoxifen (*17*). No other sites of adenine adduct formation were observed. The adenine adduct generated a stronger STOP site when adducted with activated 4-hydroxytamoxifen than α-acetoxytamoxifen. When these plasmids were used to transform bacteria, then this site formed a mutation hot spot, with activated 4-hydroxytamoxifen but not α-acetoxytamoxifen. This may indicate that for activated 4-hydroxytamoxifen the adenine adduct permits a degree of highly inaccurate translesional DNA synthesis. Alternatively, in *E. coli*, this adduct may be stalling the polymerase sufficiently to cause activation of transcription coupled error prone repair activity. Malignant breast carcinoma cell lines have been shown to have a significant error prone repair activity (*30*). Therefore, although this DNA repair pathway is much less used in nonmalignant cells, it may be of significance in some circumstances.

With activated 4-hydroxytamoxifen, but not α-acetoxytamoxifen, as the plasmid adduct level increased, a decreased

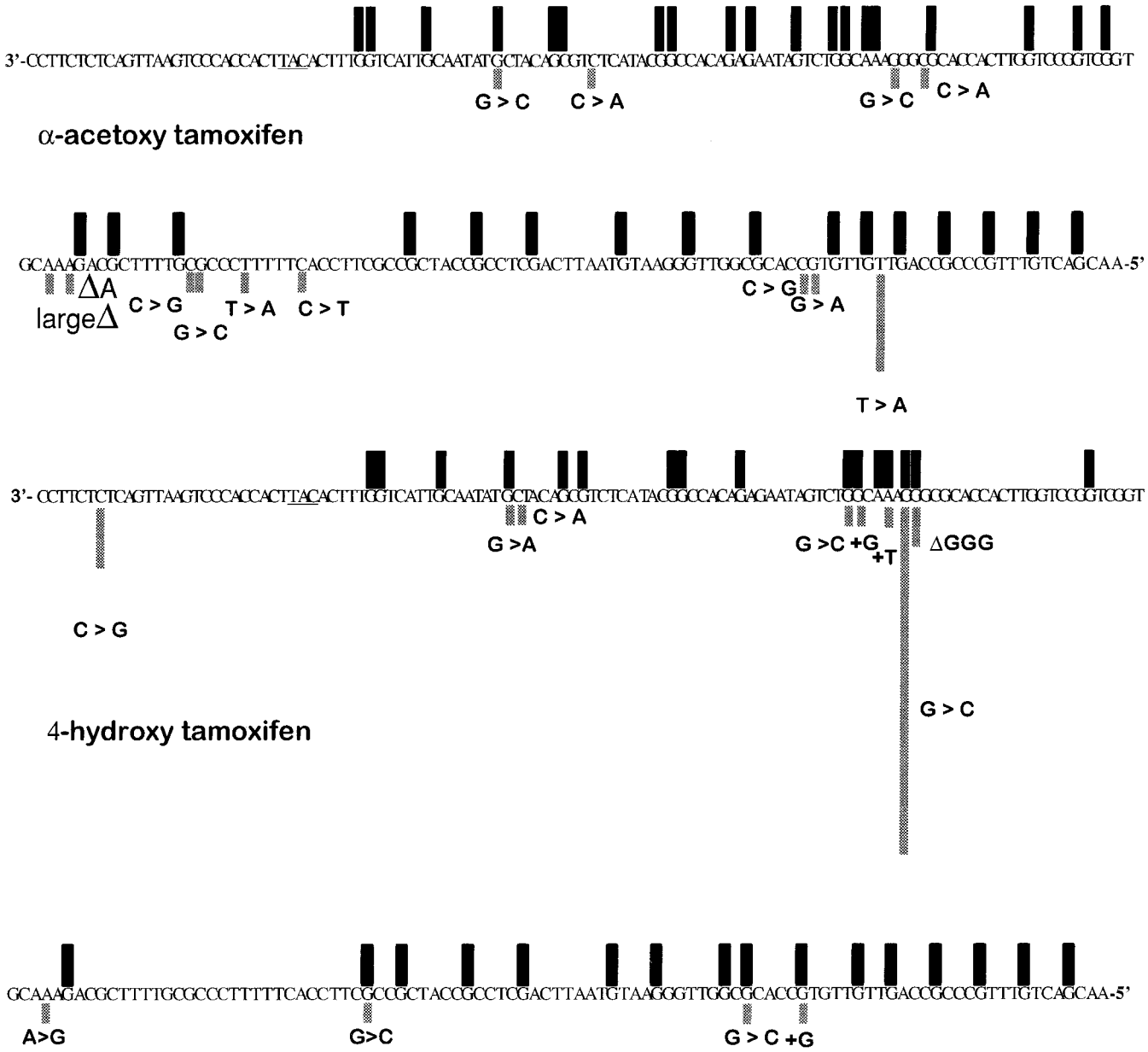

FIGURE 5: Sequencing of mutated plasmids. Mutant (blue) colonies were picked from all the plates, grown, and the plasmid prepared for sequencing use, alkaline lysis technique, and silica gel matrix. Sequencing was by a dye sublimation technique using a linear PCR amplification. The primer used was the same as that for the STOP assay. The sequence shown is the antisense (transcribed) strand in the 3′ to 5′ direction of the *lac I* gene starting at base−26 upstream of the adenosine of the translation initiation codon (underlined). This is the sequence corresponding to that "read" for the STOP assay. Only mutations detected within the region that corresponded to sequence analyzed for the STOP assay are shown. The black bars above the sequence mark the STOP sites shown in Figure 3. The gray bars show sites of mutation with the type of mutation marked. The length of the bar indicated the number of times a mutation was observed with the shortest bars corresponding to one observation.

level of bacterial transformation ability was observed (data not shown). A similar phenomenon has been reported previously with aminofluorene (AF) and *N*-acetylaminofluorene (AAF) where AAF causes a 30-fold reduction in transformation ability with in vitro adducted plasmids compared to AF (*31*). For aminofluorenes, the phenomenon is related to the greatly reduced ability of AF to block DNA polymerase progression, compared with AAF. Inhibition of DNA and, in particular, RNA polymerase progression is likely to be a cytotoxic, rather than mutagenic, lesion. This finding is reflected in our data where α-acetoxytamoxifen adducts were less able to inhibit DNA polymerase progression than those formed by activated 4-hydroxytamoxifen. Therefore, the α-acetoxytamoxifen adducts are less likely to disrupt transcription of the plasmid ampicillin resistance gene, and plasmid replication, than adducts arising from activated 4-hydroxytamoxifen. Hence, we did not see the same decrease in transformation efficiency with the α-acetoxytamoxifen adducted plasmid as that we observed with activated 4-hydroxytamoxifen.

Increased *E. coli* mutation frequency was seen with both the α-acetoxytamoxifen and the 4-hydroxytamoxifen mutated plasmids. Overall, there was about a 2 order of magnitude greater mutation frequency observed with the activated 4-hydroxytamoxifen treated plasmids than with those treated with α-acetoxytamoxifen, even after taking into account the increased mutation frequency due to the action of the HRP/H_2O_2 activation system alone.

Table 1: Mutations in Daughter pLIZ (first 500 bp) Plasmids after Extraction from "Blue" SCS-8 *Escherichia coli*[a−c]

α-acetoxytamoxifen pLIZ mutations					
experimental			controls		
pos	mutation	occurrence	pos	mutation	occurrence
23	G>C	1		no detected mutation	4
33	C>A	1	94	G>A	1
64	G>C	1	116	C>G	1
67	C>A	1	304	C>A	1
91−108	deletion	1	327	C>A	2
93	ΔA	1	475	C>G	1
104	C>G	1			
105	G>C	1			
110	T>A	1			
114	C>T	1			
159	C>G	1			
160	G>A	1			
16	T>A	5			
240−256	deletion	1			
244	G>A	1			
315	C>G	2			
388	+G	1			
441	A>T	1			
	no detected mutation	6			
4-hydroxytamoxifen pLIZ mutations					
−20	C>G	3		No detected mutations	9
22	G>A	1	30	C>T	1
23	C>A	1	31	G>T	1
58	G>C	1	460	T>G	1
59	+C	2			
62	+T	1			
64−66	ΔGGG	2			
64	G>C	18			
92	A>G	1			
121	G>C	1			
155	G>C	1			
160	+G	1			
203	ΔG	1			
244	G>A	1			
248	+A	1			
289	+C	1			
298	+C	1			
319	+T	1			
331	C>A	1			
347	G>A	1			
375	T>A	1			
384	G>A	1			
400	+C	1			
414	C>T	1			
464	T>A	1			
490	+A	1			
HRP/H_2O_2 mutations					
29	G>T	1			
58	G>C	1			
59	ΔG	2			
202	ΔG	1			
243	C>T	2			
332	C>T	1			
345	G>A	1			
362	+T	1			
398	+C	1			
418	ΔG	1			
460	T>G	1			
491	+A	1			

[a] Numbering system assumes the adenosine of the translation initiation site of the *lac I* gene to be 1. [b] n.d. is no detected mutations. [c] Spontaneous mutations.

Although the site of adduct formation was similar for both tamoxifen metabolites, the adducts generated by activated 4-hydroxytamoxifen were more mutagenic (Figure 4). This may indicate that adducts formed by 4-hydroxytamoxifen are more readily detected by transcription coupled error prone DNA repair in this system. Alternatively, they may permit a small degree of highly inaccurate translesional DNA synthesis, which results in incorporation of the wrong base, or deletions. This greater mutation propensity, and ability to inhibit T4 DNA polymerase progression, may also be an indicator that the lesion generated by activated 4-hydroxytamoxifen is much more disruptive of the DNA structure than that generated by α-acetoxytamoxifen. The structures of tamoxifen adducts support this hypothesis where the 4-hydroxytamoxifen DNA adduct would give a different hydrogen bonding pattern to complementary DNA bases than the dG-N^2-tamoxifen adduct due to the extra OH group present in the structure (Figure 1).

The ^{32}P-postlabeling data showed that α-acetoxytamoxifen formed approximately five times as many adducts on the naked plasmid DNA compared with activated 4-hydroxytamoxifen (Figure 2), assuming similar labeling efficiencies of the adducted nucleotides by T4 polymerase. As dG-N2-tamoxifen adduct standards are unavailable in the 3′ monophosphate form, and dG-N2-4-hydroxytamoxifen has never been isolated, we are unable to test T4 labeling efficiency for each adducted species and have, therefore, to assume it is similar. With this caveat however, α-acetoxytamoxifen mutation frequency was much lower than that caused by 4-hydroxytamoxifen. Therefore, there was no correlation between adduct number and propensity to cause mutations. These data clearly demonstrate that the gross number of total adducts may not be valid as a direct measure of mutagenic potential in a biological system. The adduct types formed by activated 4-hydroxytamoxifen in vitro correlate only with the minor adducts formed by tamoxifen in vivo (*14*). However, if these minor adducts were particularly potent at causing mutation, they may have a contributing effect to the mutagenic potential of tamoxifen disproportionate to their total amount.

Thus, we have shown that tamoxifen−DNA adducts can be specifically detected by the T4 DNA polymerase STOP assay, and that in vitro the major site of adduct formation is with guanines. However, site-specific formation of an adenine adduct was detected in the *lacI* gene incubated with activated 4-hydroxytamoxifen or α-acetoxytamoxifen. The adenine adduct was located between two runs of guanines, and this area of the gene was a hot spot for adduct formation, and mutation in *E. coli.* Despite α-acetoxytamoxifen forming proportionally more adducts than activated 4-hydroxytamoxifen these adducts were less inhibitory to the progression of T4 DNA polymerase, and less mutagenic. This is in accordance with the anticipated structure of the adducts that would suggest that those formed by activated 4-hydroxytamoxifen would be more disruptive to the DNA structure due to the additional hydroxyl group than those formed by α-acetoxytamoxifen. The ability of T4 DNA polymerase to bypass DNA adducts formed by α-acetoxytamoxifen, but much less so those formed by 4-hydroxytamoxifen, indicates that the mutagenic potential of 4-hydroxytamoxifen DNA adducts is probably related to a small level of highly inaccurate translesional DNA synthesis. Alternatively, the

mutations may arise due to transcription coupled error prone repair of the DNA. Although more translesional DNA synthesis is permitted by α-acetoxytamoxifen adducts this DNA synthesis may occur with a greater fidelity, resulting in a decreased frequency of mutation as well as less cytotoxicity. Finally, these data indicate the lack of relationship between the gross adduct number and mutagenic potential, and highlight the importance of assessing the possible disproportionate effect of minor adduct formation on mutagenesis.

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