DNA Adducts Formed from 4-Hydroxytamoxifen Are More Mutagenic than Those Formed by α-Acetoxytamoxifen in a Shuttle Vector Target Gene Replicated in Human Ad293 Cells†

Keith I. E. McLuckie,*,‡ Michael N. Routledge,§ Karen Brown,‡ Margaret Gaskell,‡ Peter B. Farmer,‡ Gordon C. K. Roberts,|| and Elizabeth A. Martin‡,⊥

Cancer Biomarkers and Prevention Group, The Biocentre, University of Leicester, University Road, Leicester, LE1 7RH, U.K., Molecular Epidemiology Unit, Epidemiology and HSR, School of Medicine, University of Leeds, Leeds, LS2 9JT, U.K., and Biological NMR Centre, Department of Biochemistry, Maurice Shock Medical Sciences Building, University of Leicester, University Road, Leicester, LE1 9HN, U.K.

Received January 22, 2002; Revised Manuscript Received May 13, 2002

ABSTRACT: The drug tamoxifen, used to treat breast cancer, causes liver cancer in rats and endometrial cancer in women. Tamoxifen forms liver DNA adducts in both short- and long-term dosing of rodents, and DNA adducts have also been reported in tissues of women undergoing tamoxifen therapy. It is not known if the induction of endometrial cancer in women is through these DNA adducts or through the estrogenic nature of the drug. In this study, we have investigated the mutagenicity of two model reactive intermediates of tamoxifen, α-acetoxytamoxifen and 4-hydroxytamoxifen quinone methide (4-OHtamQM). These form the same DNA adducts as those found in tamoxifen-treated rats. The two compounds were used to treat the pSP189 plasmid containing the *supF* gene, which was replicated in Ad293 cells before being screened in indicator bacteria. Plasmid reacted with 4-OHtamQM was more likely to be mutated (2−7-fold increase) than that reacted with α-acetoxytamoxifen, despite having a lower level of DNA damage (12−20-fold less), as assayed by ^{32}P-postlabeling. The two compounds induced statistically different mutation spectra in the *supF* gene. The majority of mutations in α-acetoxytamoxifen-treated plasmid were GC →TA transversions while GC→AT transitions were formed in 4-OHtamQM-treated plasmid. 4-OHTamQM-treated DNA induced a larger proportion of multiple mutations and large deletions compared to α-acetoxytamoxifen. Sites of mutational hotspots were observed for both compounds. In conclusion, the quantitatively minor DNA adduct of tamoxifen (dG-N^2-4-hydroxytamoxifen) is more mutagenic than the major tamoxifen DNA adduct (dG-N^2-tamoxifen).

Tamoxifen [*trans*-(*Z*)-1-[4-[2-(dimethylamino)ethoxy]phenyl]-1,2-diphenyl-1-butene] is an antiestrogenic drug used in the adjuvant therapy of breast cancer. 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. This was based on the finding that tamoxifen achieved a statistically significant 49% reduction in the incidence of invasive breast cancer in women with increased risk of the disease (*1*). However, both chemopreventative and therapeutic dosing strategies have been shown to slightly increase the risk of endometrial cancer in comparison to controls (*2, 3*). In addition, both short-term and long-term treatment of rats with tamoxifen results in the induction of hepatocellular carcinomas preceded by the formation of large amounts of hepatic DNA adducts (*4*−*6*). Tamoxifen DNA adducts have also been reported in leukocytes and endometrial tissue of women undergoing tamoxifen therapy (*7, 8*), adding to concerns over the long-term health hazards of tamoxifen.

In rat liver, DNA damage is initiated only after metabolic activation of tamoxifen (*9*). The principal metabolic pathway in vivo involves α-hydroxylation (*10*−*13*) followed by hydroxysteroid sulfotransferase mediated sulfate conjugation (*14, 15*). Loss of this sulfate moiety leaves a carbocation (*16*), promoting nucleophilic attack by the N^2-amino group of deoxyguanosine to form α-(deoxyguanosin-N^2-yl)tamoxifen (dG-N^2-tam)[1] (*17, 18*) (Figure 1). This adduct exists as four diastereoisomers, with the trans-forms accounting for one of the major adducts in rat liver DNA. While α-hydroxytamoxifen can react directly with DNA, the synthetic *O*-sulfonate gives greater than a 180-fold increase in adduct yield. However, the α-sulfonate of tamoxifen is very short-lived, so a model ester, α-acetoxytamoxifen, is often used for studies in vitro. Incubation of DNA with α-acetoxytamoxifen in vitro produces the same dG-N^2-tamoxifen adducts formed in rats and humans after treatment with tamoxifen (*19, 20*).

† This work supported by the Medical Research Council, U.K.

* To whom correspondence should be addressed. Phone: +44 (0)116 2231828, Fax: +44 (0)116 2231840, E-mail: kiem1@le.ac.uk.

‡ The Biocentre, University of Leicester.

§ School of Medicine, University of Leeds.

|| Biological NMR Centre, University of Leicester.

⊥ Present address: Genetic Toxicology Department, AstraZeneca, Alderley Park, Macclesfield, SK10 4TG, U.K.

[1] Abbreviations: 4-OHtamQM, 4-hydroxytamoxifen quinone methide; dG-N^2-4-hydroxytamoxifen, α-(N^2-deoxyguanosinyl)-4-hydroxytamoxifen; dG-N^2-tamoxifen, α-(N^2-deoxyguanosinyl)tamoxifen.

10.1021/bi025575i CCC: $22.00
Published on Web 06/20/2002

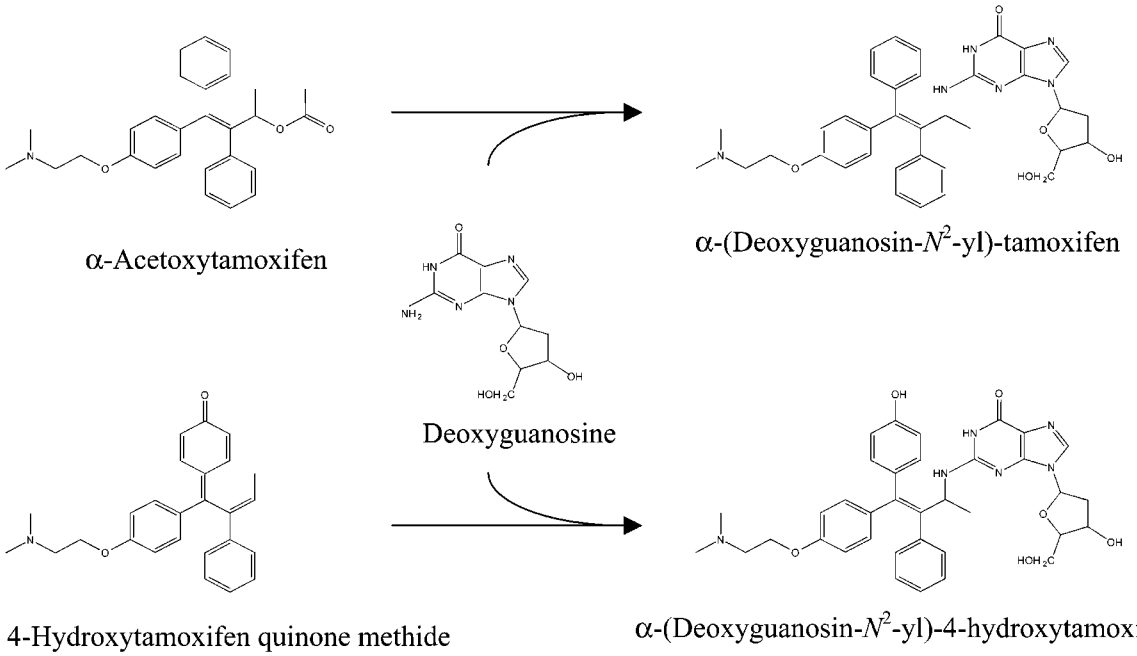

FIGURE 1: Reaction of α-acetoxytamoxifen with deoxyguanosine yields the major α-(deoxyguanosin-N^2-yl)tamoxifen adduct in rats. Reaction of 4-hydroxytamoxifen quinone methide with deoxyguanosine yields a minor adduct, α-(deoxyguanosin-N^2-yl)-4-hydroxytamoxifen, in rats.

A second major tamoxifen metabolite, 4-hydroxytamoxifen (*21*), is also thought to be activated to a DNA reactive species either via α-hydroxylation, presumably followed by α-sulfation, or possibly via formation of a quinone methide (*22*). Studies in rats have reported the formation of DNA adducts derived from 4-hydroxytamoxifen (*23*) although in rat liver these have been detected at much lower levels than those derived from α-hydroxytamoxifen (*24*).

Recent studies have shown that site-specific dG-N^2-tamoxifen adducts induce primarily GC→TA transversions in COS-7 cells (*25*). In addition, administration of tamoxifen causes GC→TA transversions in both the *cII* and *lacI* genes in lambda/*lacI* transgenic rats (*26, 27*). Although most efforts to date have focused on the major dG-N^2-tamoxifen adducts, we have previously reported that adducts formed by 4-hydroxytamoxifen are 2 orders of magnitude more mutagenic in *E. coli* than those arising from α-acetoxytamoxifen (*28*).

To compare the mutagenicity of tamoxifen DNA adducts replicated in human cells, we have used the *supF* forward mutation assay to study mutations induced by α-acetoxytamoxifen and 4-hydroxytamoxifen quinone methide (4-OH-tamQM). The *supF* assay (*29, 30*) has been widely used to study the mutagenicity of compounds that form bulky adducts when reacted with DNA. The assay detects 97% of possible base substitutions within the 85 base pair *supF* gene (*31*) as well as deletions and insertions, and because the plasmid is treated in vitro, aliquots of the treated DNA can be analyzed for adduct quantification in parallel to the mutation assay. To this end, a previously developed ^{32}P-postlabeling method for analysis of tamoxifen DNA adducts was used (*24*). The results we report here show that minor tamoxifen DNA adducts may contribute significantly to the mutagenicity of the compound in mammalian cells.

MATERIALS AND METHODS

Materials. The trans isomer of α-hydroxytamoxifen was synthesized using the method described by Foster and coworkers (*32*). *trans*-α-Acetoxytamoxifen was prepared from *trans*-α-hydroxytamoxifen using the published method (*17*). [γ-^{32}P]ATP (>185 TBq/mmol, >5000 Ci/mmol, 370 MBq/mL) was purchased from Amersham, Buckinghamshire, U.K. T4 polynucleotide kinase (3′-phosphatase free) and calf spleen phosphodiesterase were bought from Roche, Lewes, East Sussex, U.K. All other chemicals were from Sigma, Poole, Dorset, U.K., unless otherwise stated.

Shuttle Vector Plasmid, Bacterial Strain, and Cell Lines. The plasmid pSP189 containing the *supF* gene (*30*) and *E. coli* strain MBM7070 were gifts from M. Seidman, Oncor Pharmaceuticals, Gaithersburg, MD. Human embryonic adenovirus-transformed kidney cells (Ad293) were cultured from cells previously provided by Dr. A. Dipple, National Cancer Institute, Frederick, MD. Ad293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Life Technologies Ltd., Paisley, U.K.) at 37 °C in 5% CO_2 in air.

Treatment of DNA with α-Acetoxytamoxifen. Aliquots of pSP189 plasmid (200 μg, in 200 μL of Tris−EDTA buffer, pH 8.0) were incubated with 10, 25, and 50 μM α-acetoxytamoxifen in ethanol at 37 °C for 18 h. A control DNA incubation, to which only ethanol was added, was also carried out. The samples were extracted with 3 × 400 μL of water-saturated ethyl acetate to remove unreacted α-acetoxytamoxifen. Plasmid DNA was then precipitated with 3 M sodium acetate/ice-cold ethanol and redissolved in 200 μL of sterile tissue culture grade water.

Treatment of DNA with 4-Hydroxytamoxifen Quinone Methide. 4-Hydroxytamoxifen (6.52 mg) was activated to a quinone methide with silver(II) oxide (42.92 mg in 1.8 mL of dry chloroform, stirred for 30 min) using established procedures (*33*). The reaction mixture was filtered and dried to a yellow−brown residue under nitrogen. This was dissolved in ethanol/acetonitrile (1:1 v/v), and added to 100 μg of pSP189 plasmid. Final concentrations of 0, 50, 100, and 250 μM were used. After incubation at 37 °C for 18 h,

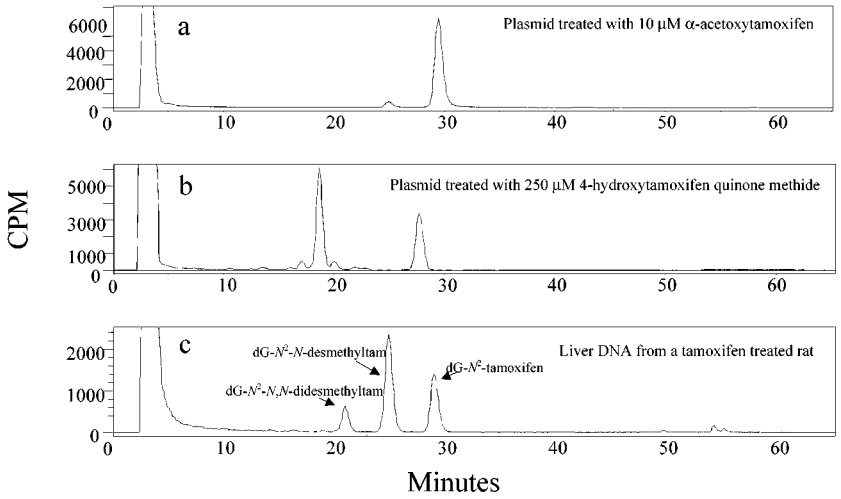

FIGURE 2: Representative radioactive HPLC chromatograms obtained for plasmid treated with α-acetoxytamoxifen (a), 4-hydroxytamoxifen quinone methide (b), and liver DNA from a tamoxifen-treated Wister Han Rat (40 mg/kg tamoxifen in diet for 6 months)(c).

the unreacted 4-hydroxytamoxifen quinone methide was extracted from the plasmid with diethyl ether (5 × 400 μL). Plasmid DNA was precipitated with 3 M sodium acetate/ice-cold ethanol and redissolved in 200 μL of sterile tissue culture grade water.

^{32}P-Postlabeling of α-Acetoxytamoxifen or 4-Hydroxytamoxifen Quinone Methide-Treated Plasmid. To quantify the number of tamoxifen DNA adducts on the treated plasmids, aliquots of plasmid DNA (5 μg) were analyzed by the ^{32}P-postlabeling assay, incorporating a nuclease P1 enhancement step. ^{32}P-Postlabeled nucleotides were separated by HPLC and measured by on-line radiochemical detection (*24*).

Transfection and Transformation. Subconfluent cells were transfected with α-acetoxytamoxifen- or 4-OHtamQM-treated plasmid (10 μg per 9 cm culture plate) using the calcium phosphate precipitation technique (*34*). After 48 h, plasmid was recovered using plasmid purification kits (Qiagen, Crawley, West Sussex). Aliquots of recovered plasmid were used to transform electrocompetent MBM7070 *E. coli* by electroporation using Gene Pulser apparatus (Biorad, Hercules, CA). Transformants were plated onto LB agar plates containing ampicillin (100 μg/mL), 5-bromo-4-chloro-3-indolyl-β-D-galactose (X-gal) (75 μg/mL), and isopropyl-β-D-thiogalactoside (IPTG) (25 μg/mL). Mutant colonies were white when grown on X-gal-containing media, whereas wild-type colonies were blue.

Sequencing. Plasmid was extracted from white mutant colonies using plasmid purification kits (Qiagen, Crawley, West Sussex) and sequenced using the primer 5′-GGCGA-CACGGAAATGTTGAA-3′ (Protein and Nucleic Acid Chemistry Laboratory, Hodgkin Building, University of Leicester, U.K.). The pSP189 shuttle vector contains an 8 base 'signature sequence' giving 4^8 (65 536) possible unique sequences (*30, 31*). Any mutants with a duplicated 'signature' were excluded from further analysis. Poisson distribution analysis was used to assess the randomness of spectra. Hotspots were assumed when the number of mutations observed was 4-fold or more greater than the number expected for a random (Poisson) distribution.

RESULTS

^{32}P-Postlabeling Analysis of Plasmid pSP189 Treated with α-Acetoxytamoxifen or 4-Hydroxytamoxifen Quinone Methide. To assess the mutagenic effects of tamoxifen reactive intermediates derived from α-hydroxytamoxifen and 4-hydroxytamoxifen, the shuttle vector plasmid pSP189 was modified by in vitro reaction with α-acetoxytamoxifen and 4-OHtamQM, respectively. ^{32}P-Postlabeling analysis of the modified plasmids revealed HPLC adduct profiles similar to previous reports for each compound as illustrated in Figure 2 (*24, 28*). As judged by HPLC, treatment with α-acetoxytamoxifen results in the formation of the dG-N^2-tamoxifen DNA adduct that has previously been detected in liver tissue of tamoxifen-dosed rats (Figure 2a). An additional minor peak is also observed eluting just prior to the major adduct, which, based on retention time, is the N-demethylated dG-N^2-tamoxifen adduct (Figure 2c) (*35*). Following incubation of 4-OHtamQM with plasmid DNA, two main ^{32}P-postlabeled adduct peaks are detected (Figure 2b). Although these peaks do not coelute with any adduct peaks detected in DNA from rat liver tissue shown in Figure 2c, we have previously demonstrated the presence of up to 12 adduct peaks in similarly treated rats, and 1 of these coelutes with the main 4-hydroxytamoxifen-derived adduct (*24*). The major products of the reaction of 4-OHtamQM, produced by chemical oxidation, with DNA are known to be isomers of a 4-hydroxylated form of dG-N^2-tam (*22*). This reactive intermediate can also be generated enzymatically. We have previously shown that incubation of 4-hydroxytamoxifen with horseradish peroxidase yields one major ^{32}P-postlabeled adduct peak, which corresponds to the larger of the two adduct peaks observed in this study (*24, 28*). This difference in adduct profile is probably due to the different methods used to activate 4-hydroxytamoxifen. The additional adduct, which is present at low levels with peroxidase activation, may be an isomer of dG-N^2-4-hydroxytam or may be an as yet unidentified adduct. Quantification of adduct levels over the concentration range used demonstrated a dose-dependent increase in DNA damage for each compound (Table 1). Higher concentrations of 4-OHtamQM were used in the incubations compared to α-acetoxytamoxifen, as the former is known to generate lower levels of adducts (*28*). In the present study, 4-OHtamQM (50 μM) induced a 50-fold lower level of DNA adducts than an equimolar dose of α-acetoxytamoxifen. The degree of pSP189 modification by α-acetoxytamoxifen equates to a level of 0.5, 1.6, or 2.5

Table 1: Mutation Frequency and Adduct Number Induced by α-Acetoxytamoxifen and 4-Hydroxytamoxifen

treatment	mutation frequency[a]	adduct number[b] (±SD)
	α-Acetoxytamoxifen	
control[c]	6.6	0 ± 0
10 μM	10.5	50 ± 8.8
25 μM	9.3	160 ± 8
50 μM	32.3	240 ± 52
	4-Hydroxytamoxifen	
control[c]	3.3	0 ± 0
50 μM	6.2	5 ± 0.6
100 μM	64.4	8 ± 2.3
250 μM	72.1	20 ± 1

[a] Mutation frequency per 10^4 colonies. [b] Adduct number per 10^6 nucleotides. [c] Mutation frequency of plasmid dissolved in water only was 0.2×10^{-4}.

Table 2: Types of Sequence Alterations in the *supF* Gene of pSP189 Plasmids Treated with α-Acetoxytamoxifen and 4-Hydroxytamoxifen Quinone Methide

	number of plasmids with mutations (%)	
types	α-acetoxytam	4-hydroxytam
base substitutions	132 (90)	96 (62)
single	109 (74)	49 (32)
tandem	3 (2)	1 (0.6)
multiple	20 (14)	46 (30)
frameshifts	15 (10)	58 (38)
single base deletion	3 (2)	2 (1.3)
>2 base deletion	12 (8.2)	50 (32)
single base insertion	0 (0)	3 (1.9)
>2 base insertion	0 (0)	3 (1.9)
total plasmids sequenced	147 (100)	154 (100)

adducts per plasmid (4952 base pairs) for the 10, 25, and 50 μM doses, respectively. The three treatments with 4-OHtamQM (50, 100, and 250 μM) induced approximately 0.05, 0.1, and 0.2 adducts per plasmid.

Mutation Frequency in the supF Gene. While the spontaneous mutation frequency of plasmid suspended in water (2 in 10^5) was similar to data previously published (*36*), the solvent-treated control plasmid did show increased mutation frequency in the absence of α-acetoxytamoxifen or 4-OHtamQM (Table 1). Table 1 also shows that the total mutation frequency increased with dose, and hence adduct level, after treatment with both α-acetoxytamoxifen and 4-OHtamQM. The 4-OHtamQM induced a 2−7-fold greater increase in mutation frequency than treatment with α-acetoxytamoxifen, even though the number of adducts produced by 4-OHtamQM treatment was 10−20-fold lower than by α-acetoxytamoxifen treatment.

Mutation Types Found in the supF Gene. White mutant colonies were collected, and the *supF* gene was sequenced to identify the types of mutation and their location within the gene. The majority of mutations induced by both α-acetoxytamoxifen and 4-OHtamQM were base substitutions (90% and 62% of all mutants, respectively) as shown in Table 2. In α-acetoxytamoxifen-treated cells, most of these were in the form of single base substitutions (74%), with a few tandem (2%) and a larger proportion of multiple substitutions (14%, 2 or more substitutions at nonadjacent sites along the *supF* gene). In 4-OHtamQM-treated cells, there was an almost equal amount of single and multiple substitutions (30% and 32%, respectively) along with a single tandem substitution (0.6%). Frameshift mutations were relatively infrequent in α-acetoxytamoxifen-treated cells (10%) but more abundant in 4-OHtamQM-dosed cells (38%). Of these frameshifts, insertions were uncommon for both treatments (0−3.8%). Deletions, especially in the form of large deletions (greater than two adjacent bases deleted), were more common. Treatment with α-acetoxytamoxifen induced 2% single base deletions and 8.2% large (greater than 2 bases) deletions. Treatment with 4-OHtamQM induced 1.3% single base deletions and a high number (32%) of large (greater than 2 bases) deletions.

Figure 3 illustrates the effect of increasing dose on the mutation profiles induced by α-acetoxytamoxifen and 4-OHtamQM. Each type of substitution is expressed as a percentage of the total substitutions detected for each dose and corrected relative to the mutation frequency observed at the highest dose for each treatment. For α-acetoxytamoxifen-modified plasmid, as the dose increases so does the proportion of transversions compared to transitions. Overall, the most common single base substitutions are GC→TA transversions, occurring over 2-fold more frequently than GC→AT transitions (Figure 3). The mutation pattern for this compound is consistent with the fact that α-acetoxytamoxifen binds predominantly to deoxyguanosine; therefore, most substitutions would be expected to occur at GC base pairs. In α-acetoxytamoxifen-treated plasmids with multiple mutations in the *supF* gene, the predominant substitutions are GC→AT transitions, followed by GC→TA transversions. In the single base substitutions induced by 4-OHtamQM treatment, most of the mutations are at GC base pairs, with the major substitution, GC→AT transitions, up to 2-fold more prevalent than GC→TA transversions. For multiple mutations, the number of GC→TA transversions slightly exceeds the number of GC→AT transitions.

Mutation Spectra in the supF Gene. The distribution of base substitution mutations within the *supF* gene for plasmids dosed with α-acetoxytamoxifen and 4-OHtamQM is shown in Figure 4. Multiple base substitutions, which are illustrated on separate spectra, were included due to the large proportion induced by 4-OHtamQM; almost one-third of all mutants had multiple mutations. When these spectra are compared using the Hyperg program (*37*), they are all found to be significantly different from each other. There are six hotspots in the spectrum of single base substitutions induced by α-acetoxytamoxifen. A hotspot is defined as a site where the number of mutations observed was 4-fold or more greater than the number expected for a random Poisson distribution. These are at positions 105, 118, 122, 159, 160, and 163, all of which are at GC sites. In the spectrum of multiple α-acetoxytamoxifen substitutions, there are two definite hotspots at positions 156 and 168 and two more 'borderline' hotspots at positions 155 and 174, all of which are at GC sites. Four hotspots are apparent in the spectrum of single base substitutions induced by 4-OHtamQM at positions 129, 139, 155, and 156. The multiple base substitution spectrum has four hotspots at positions 100, 133, 156, and 174. All of these hotspots are also at sites of GC base pairs. Positions 155, 156, and 174 had hotspots in two or more of the spectra.

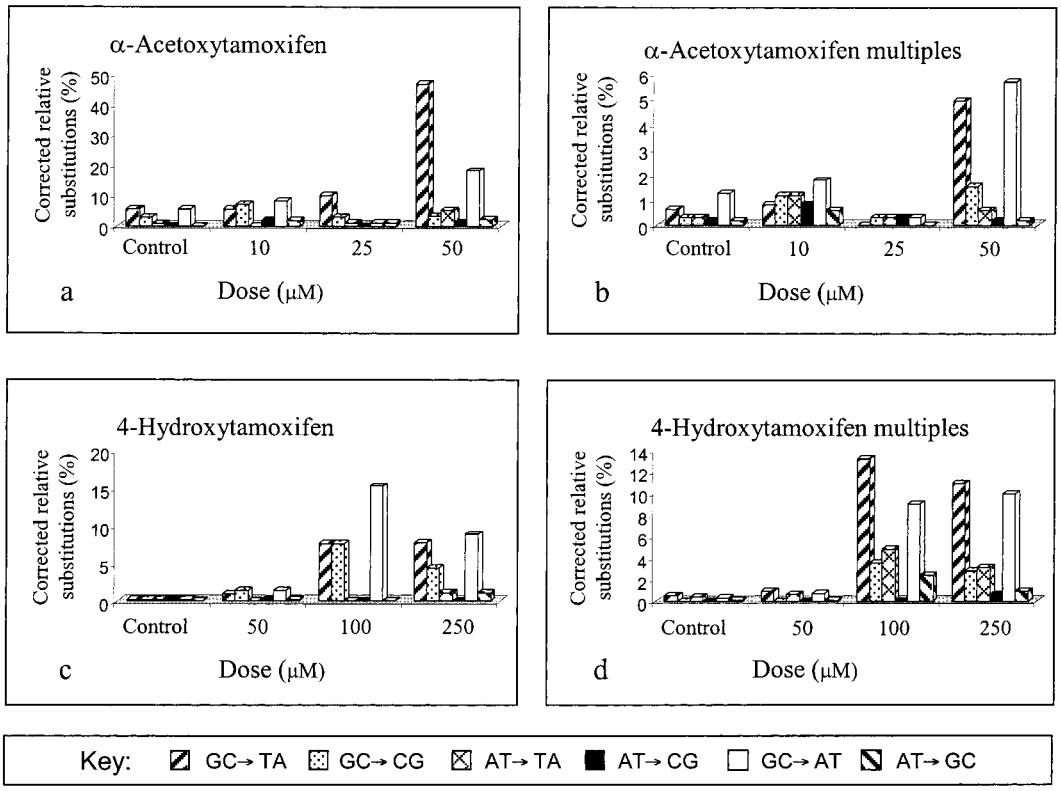

FIGURE 3: Bar charts illustrating the relative amounts of the different base substitutions (corrected for mutation frequency) for pSP189 plasmid treated with α-acetoxytamoxifen (a, single base substitutions; b, multiple substitutions) and 4-hydroxytamoxifen quinone methide (c, single base substitutions; d, multiple substitutions).

DISCUSSION

This paper presents a comparison of the patterns of mutagenesis induced in the *supF* gene by two tamoxifen derivatives, α-acetoxytamoxifen and 4-OHtamQM. α-Acetoxytamoxifen generates the major DNA adducts formed in rat liver (*19*), while the reactive quinone methide of the metabolite 4-hydroxytamoxifen is thought to account for the formation of minor adducts found in rat liver (*23, 24*). Since little is known about the mutagenic potential of tamoxifen adducts other than the major dG-N^2-tam adduct, a primary aim of this study was to evaluate the relative contribution of different types of tamoxifen adducts to the mutagenicity of this drug in human cells. The *supF* gene has been used as the 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 that are induced (*38*−*40*). In this study, we have looked at the mutation spectra induced when treated plasmid was replicated in human adenovirus transformed kidney (Ad293) cells.

Replication of adducted plasmid in human Ad293 cells resulted in an increase in mutation frequency above background control levels for both tamoxifen derivatives. Furthermore, mutation frequency increased with higher plasmid adduct levels, which is in contrast to our earlier study in *E. coli* that reported a lack of correlation between total adduct number on the pLIZ lambda shuttle vector and mutagenicity in the *lacI* gene (*28*). In the present study, for each compound, at the lower doses used there was only a small increase in mutation frequency above the solvent control. With higher doses, inducing above 160 adducts per 10^6 nucleotides (1.6 adducts per plasmid) in α-acetoxytamoxifen-treated plasmid and 8 adducts per 10^6 nucleotides (0.08 adducts per plasmid) in 4-OHtamQM-treated plasmids, there is a sharp increase in mutation frequency. This may suggest there is a threshold level of tolerable damage which the cells are able to repair. Low levels of DNA damage will induce DNA repair, and adducts will be removed. Higher levels may saturate the available repair mechanisms, resulting in an increase in mutation frequency. The fact that 4-OHtamQM induced such a marked increase in mutation frequency in human cells compared to α-acetoxytamoxifen at the two higher treatment doses suggests that the dG-N^2-4-hydroxytamoxifen DNA adduct is a significantly more mutagenic lesion, which may deserve more consideration than it has so far received. The increased mutagenicity of 4-hydroxytamoxifen-induced adducts is thought to be a consequence of the increased hydrogen-bonding potential of dG-N^2-4-OHtam over dG-N^2-tam due to the presence of the 4-hydroxy group (*28*). Extra interactions with complementary bases may disrupt DNA structure, resulting in lesions which could be more readily detected by transcription-coupled error-prone DNA repair or highly inaccurate translesional DNA synthesis, resulting in the incorporation of wrong bases.

Another important feature of the tamoxifen DNA adducts investigated in this study is the low adduct levels needed to induce mutagenesis above background, compared to previous reports for carcinogen adducts. Previously, mutation frequencies similar to those reported here (i.e., around 1×10^{-3}), with 0.05 adducts per plasmid, have been reported for *supF* plasmid containing acetylaminofluorene (*41*), benzo[*a*]pyrene diol epoxide (*42*), or 1,6-dinitropyrene adducts (*43*), replicated in the same cell line (with the same repair proficiency), but containing between 20 and 460 times as many adducts per plasmid as the 4-hydroxytamoxifen. In our experiments,

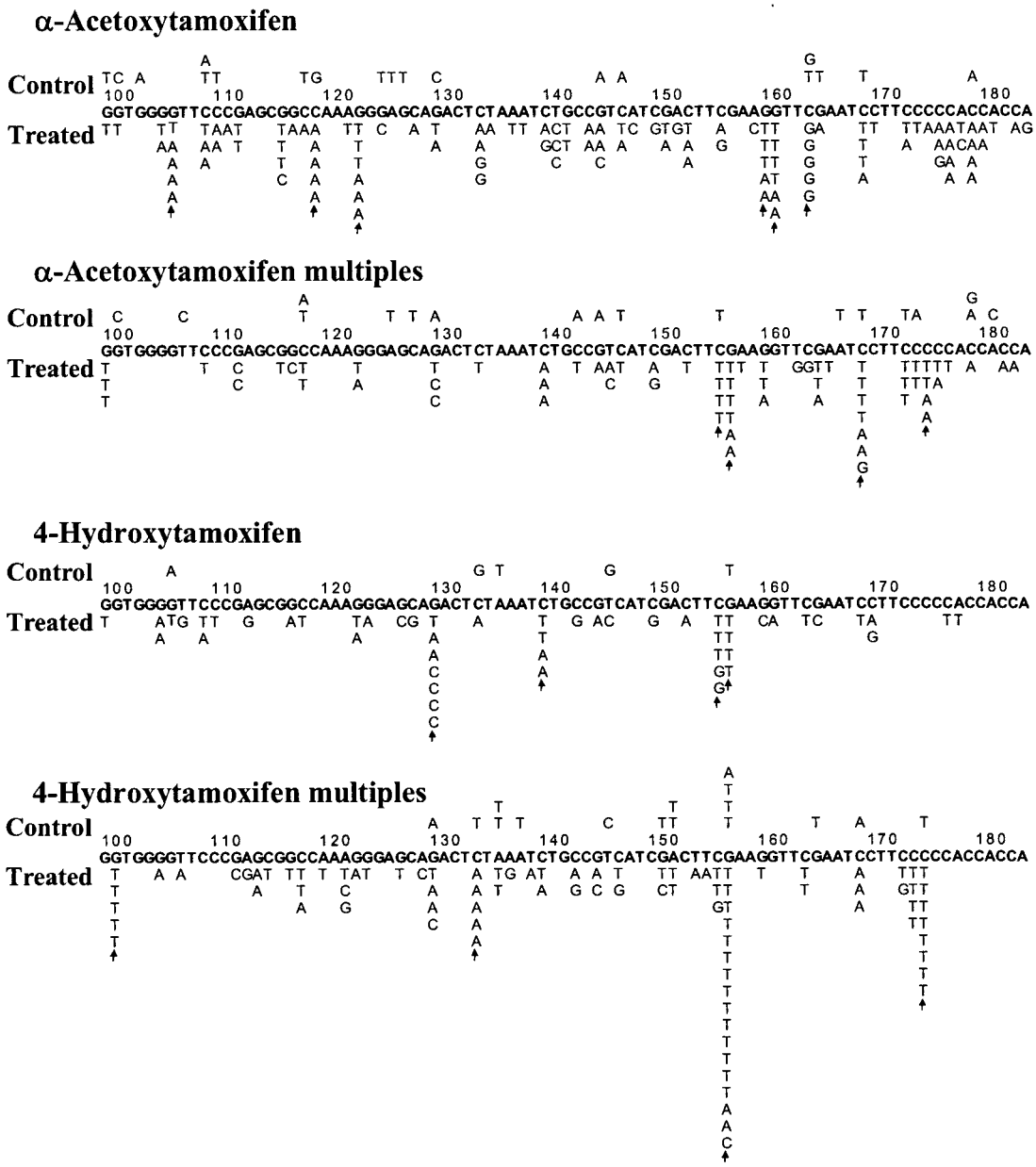

FIGURE 4: Mutation spectra induced in Ad293 cells by α-acetoxytamoxifen and 4-hydroxytamoxifen quinone methide. The 5′ to 3′ sequence of the transcribed strand of the wild-type *supF* gene is shown, with letters below the wild-type sequence indicating the position and type of point mutations induced by treatments and the letters above the wild-type sequence indicating the position and type of point mutations induced in the control samples. The single base substitution spectra comprise all three doses, while multiple base substitutions were from a single dose (50 μM for α-acetoxytamoxifen and 250 μM for 4-hydroxytamoxifen quinone methide). Arrows denote hotspot sites.

α-acetoxytamoxifen showed a similar mutagenicity, on an adduct per plasmid basis, as has been reported for benzo[*a*]pyrene diol epoxide (*42*).

Previously reported mutation spectra in Ad293 cells (*44*, *45*), human lymphoblasts (*46*), and monkey kidney cells (*47*) have shown that the GC → AT transition is the preferred spontaneous mutation. In this investigation, GC → TA transversions are just as prevalent as GC → AT transitions in control plasmids, and GC → CG transversions are only slightly less so. Upon treatment, the GC → AT transition becomes the major mutation for α-acetoxytamoxifen multiple substitutions and 4-OHtamQM single base substitutions. The GC → TA transversion becomes the preferred mutation in α-acetoxytamoxifen single base substitutions and 4-OHtamQM multiple substitutions. Miscoding of the damaged DNA consistent with induction of the GC → TA transversion has been demonstrated for the dG-N^2-tamoxifen adduct in site-specifically-modified oligonucleotides replicated in vitro (*48*). Besides misincorporation of A opposite the dG-N^2-tamoxifen adduct, misincorporation of G and, in some sequences, T was also shown to be possible, albeit at a lower frequency. For both treatments, all hotspots were at GC base pair sites, presumably as a result of adduct formation on deoxyguanosine. There was a noticeable preference for the hotspot site to be preceded and followed by a purine, particularly adenosine. Positions 155, 156, and 174 had hotspots in two or more of the spectra. Hotspots at 156 and 174 appeared in both multiple base substitution spectra, and the hotspot at 156 was present in the 4-OHtamQM single spectra. Position 156 also showed as a hotspot in the control plasmid that was incubated with either acetonitrile or ethanol only. Whether the solvent caused this is not known, although Lewis

et al. did see a hotspot at this position when grouping spontaneous mutations (*49*). Single and multiple base substitutions have been presented on separate spectra because it has been suggested that these multiple mutations arise through a different mechanism to single base mutations (*50*).

The higher percentage of plasmids with multiple mutations induced by 4-OHtamQM compared to α-acetoxytamoxifen may point to a different mechanism of mutagenesis accounting for some of the mutations induced by the former. Similarly, the increased amount of both insertions and large deletions in plasmids treated with 4-OHtamQM compared to α-acetoxytamoxifen suggests that not all mutations induced by 4-OHtamQM are due to misreading of adducted bases, but may be in part due to the formation of cross-links between bases. Previous work has shown that mitomycin C, which reacts with deoxyguanosine at the *N*-2 position like tamoxifen, induces a comparable amount of deletions to 4-OHtamQM in Ad293 cells (34% of all mutations) (*51*) along with a large amount of GC → TA transversions. Mitomycin C is used as a treatment for bladder and rectal cancers due to its DNA cross-linking ability. The anticancer drug Melphalan also causes DNA cross-linking, probably by *N-7* alkylation of two guanines on opposite DNA strands. When used to treat pZ189 plasmid containing the *supF* gene in Ad293 cells, this also induced a large proportion of deletions (16−28%) (*52*). Metabolic activation of both tamoxifen and 4-hydroxytamoxifen in rat liver microsomes results in the formation of dimers, possibly through a free radical mechanism (*53*). A possible explanation for the induction of large deletions with 4-hydroxytamoxifen-derived adducts, therefore, may be the generation of free radical species resulting in the production of tamoxifen dimer adducts which could be in the form of inter- or intrastrand cross-links. It has previously been reported that multiple mutations correlate with increased levels of single strand breaks in plasmid DNA, induced either by treatment or during repair of induced lesions in repair-competent cells (*54*). The production of free radical species could, therefore, account for this increase in both multiple mutations and deletions, with treatment by 4-OHtamQM, via the induction of strand breaks. To test these hypotheses, we are presently investigating the formation of cross-links and free radical species and their relationship with the induction of strand breaks, and deletion mutations.

Since the *supF* target gene is a double-stranded DNA molecule, it is not possible to determine absolutely which strand contained the mutagenic lesion. However, as tamoxifen does not induce significant amounts of damage at cytosine residues and it is known that the dG-N^2-tamoxifen adduct induces GC → TA mutations, it is probable that the majority of GC → TA mutations are due to misincorporation opposite a damaged G. Considering this, it is possible to review the mutation spectrum from the point of view of any possible strand bias, as the coding strand is more efficiently repaired than the noncoding strand (*55*, *56*). After treatment with 4-OHtamQM, the number of mutations presumably derived from damage to the transcribed and nontranscribed strands are almost the same (94 vs 103). After treatment with α-acetoxytamoxifen, there is less damage induced in the transcribed strand than in the nontranscribed strand (72 vs 114). This may suggest that adducts derived from α-acetoxytamoxifen are repaired more efficiently than those formed by 4-OHtamQM in this cell line. This is in contrast to previous work from this group that has shown that tamoxifen adducts are removed from rat liver DNA with no detectable difference in the rates of repair of individual adducts (*6*, Martin, E.A., et al., unpublished results). However, using an in vitro human nucleotide excision repair system, site-specific dG-N^2-tamoxifen adduct isomers in oligodeoxyribonucleotides have been shown to be differentially repaired (*57*). Overall, adducts were removed with a poor to moderate efficiency with the cis-forms being removed most efficiently. It is therefore likely that differences in the chemical structure and overall adduct profile will have a significant influence on the repair and consequently mutation spectra of tamoxifen adducts in human cells. To test this hypothesis, we are currently investigating the role of nucleotide excision repair in the mutagenesis of α-acetoxytamoxifen- and 4-OHtamQM-derived adducts.

There is some evidence that tamoxifen adducts are formed in tissues of women taking this effective anti-cancer drug (*7*, *8*). Although these adducts may be formed at low levels, we have shown here that tamoxifen DNA adducts may be highly mutagenic if formed. In particular, the adducts formed by 4-hydroxytamoxifen in the *supF* gene are, when replicated in human Ad293 cells, more mutagenic than those formed by α-acetoxytamoxifen (the major tamoxifen-DNA adducts). The two treatments induce markedly different mutation spectra and mutation types. We would conclude that the 4-hydroxytamoxifen metabolite of tamoxifen has potential to cause serious mutagenic damage to DNA. Whether this is a contributing factor in the induction of endometrial cancer in women by tamoxifen remains to be seen.

ACKNOWLEDGMENT

Dr. Michael Butterworth and Robert T. Heydon (MRC Toxicology Unit, Leicester) are thanked for their technical assistance.

REFERENCES

1. Fisher, B., Costantino, J. P., Wickerham, D. L., Redmond, C. K., Kavanah, M., Cronin, W. M., et al. (1998) *J. Natl. Cancer Inst. 90*, 1371−1388.
2. Fornander, T., Cedermark, B., Mattsson, A., Skoog, L., Theve, T., Askergren, J., Rutqvist, L. E., Glas, U., Silfversward, C., Somell, A., Wilking, N., and Hjalmar, M. (1989) *Lancet 1*, 117−119.
3. Fisher, B., Costantino, J. P., Redmond, C. K., Fisher, E. R., Wickerham, D. L., Cronin, W. M., and other National Surgical Adjuvant Breast and Bowel Project Investigators (1994) *J. Natl. Cancer Inst. 86*, 527−537.
4. Han, X., and Liehr, J. G. (1992) *Cancer Res. 52*, 1360−1363.
5. Greaves, P., Goonetilleke, R., Nunn, G., Topham, J., and Orton,T. (1993) *Cancer Res. 53*, 3919−3924.
6. Carthew, P., Martin, E. A., White, I. N. H., De Matteis, F., Edwards, R. E., Dorman, B. M., Heydon, R. T., and Smith, L. L. (1995) *Cancer Res. 55*, 544−547.
7. Hemminki, K., Rajaniemi, H., Koskinen, M., and Hansson, J. (1997) *Carcinogenesis 18*, 9−13.
8. Shibutani, S., Suzuki, N., Terashima, I., Sugarman, S. M., Grollman, A. P., and Pearl, M. L. (1999) *Chem. Res. Toxicol. 12*, 646−653.
9. Moorthy, B., Sriram, P., Pathak, D. N., Bodell, W. J., and Randerath, K. (1996) *Cancer Res. 56*, 53−57.
10. Phillips, D. H., Carmichael, P. L., Hewer, A., Cole, K. J., and Poon, G. K. (1994) *Cancer Res. 54*, 5518−5522.
11. Potter, G. A., McCague, R., and Jarman, M. (1994) *Carcinogenesis 15*, 439−442.

12. Jarman, M., Poon, G. K., Rowlands, M. G., Grimshaw, R., Horton, M. N., Potter, G. A., and McCague, R. (1995) *Carcinogenesis 16*, 683−688.
13. Phillips, D. H., Carmichael, P. L., Hewer, A., Cole, K. J., Hardcastle, I. R., Poon, G. K., Keogh, A., and Strain, A. J. (1996) *Carcinogenesis 19*, 1061−1069.
14. Shibutani, S., Dasaradhi, L., Terashima, I., Banoglu, E., and Duffel, M. W. (1998) *Cancer Res. 58*, 647−653.
15. Davis, W., Venitt, S., and Phillips, D. H. (1998) *Carcinogenesis 19*, 861−866.
16. Sanchez, C., Shibutani, S., Dasaradhi, L., Bolton, J. L., Fan, P. W., and McClelland, R. A. (1998) *J. Am. Chem. Soc. 120*, 13513−13514.
17. Osborne, M. R., Hewer, A., Hardcastle, I. R., Carmichael, P. L., and Phillips, D. H. (1996) *Cancer Res. 56*, 66−71.
18. Rajaniemi, H., Rasanen, I., Koivisto, P., Peltonen, K., and Hemminki, K. (1999) *Carcinogenesis 20*, 305−309.
19. Dasaradhi, L., and Shibutani, S. (1997) *Chem. Res. Toxicol. 10*, 189−196.
20. Shibutani, S., Ravindernath, A., Suzuki, N., Terashima, I., Sugarman, S. M., Grollman, A. P., and Pearl, M. L. (2000) *Carcinogenesis 21*, 1461−1467.
21. Randerath, K., Moorthy, B., Mabon, N., and Sriram, P. (1994) *Carcinogenesis 15*, 2087−2094.
22. Marques, M. M., and Beland, F. A. (1997). *Carcinogenesis 18*, 1949−1954.
23. Pathak, D. N., Pongracz, K., and Bodell, W. J. (1995) *Carcinogenesis 16*, 11−15.
24. Martin, E. A., Heydon, R. T., Brown, K., Brown, J. E., Lim, C. K., White, I. N. H., and Smith, L. L. (1998) *Carcinogenesis 19*, 1061−1069.
25. Terashima, I., Suzuki, N., and Shibutani, S. (1999) *Cancer Res. 59*, 2091−2095.
26. Davies, R., Oreffo, V. I. C., Martin, E. A., Festing, M. F. W., White, I. N. H., Smith, L. L., and Styles, J. A. (1997) *Cancer Res. 57*, 1288−1293.
27. Davies, R., Gant, T. W., Smith, L. L., and Styles, J. A. (1999) *Carcinogenesis 20*, 1351−1356.
28. Lowes, D. A., Brown, K., Heydon, R. T., Martin, E. A., and Gant, T. W. (1999) *Biochemistry 38*, 10989−10996.
29. Seidman, M. M., Dixon, K., Razzaque, A., Zagursky, R. K., and Berman, M. L. (1985) *Gene 38*, 233−237.
30. Parris, C. N., and Seidman, M. M. (1992) *Gene 117*, 1−5.
31. Routledge, M. N., McLuckie, K. I. E., Jones, G. D. D., Farmer, P. B., and Martin, E. A. (2001) *Carcinogenesis 22*, 1231−1238.
32. Foster, A. B., Jarman, M., Leung, O.-T., McCague, R., Leclerq, G., and Devleeschouwer, N. (1985) *J. Med. Chem. 28*, 1491−1497.
33. Liehr, J. G., DaGue, B. B., Ballatore, A. M., and Henkin, J. (1983) *Biochem. Pharmacol. 32*, 3711−3718.
34. Graham, F. L., and Van der Eb, A. J. (1973) *Virology 52*, 456−467.
35. Brown, K., Heydon, R. T., Jukes, R., White, I. N. H., and Martin, E. A. (1999) *Carcinogenesis 20*, 2011−2016.
36. Routledge, M. N., Mirsky, F. J., Wink, D. A., Keefer, L. K., and Dipple, A. (1994) *Mutat. Res. 322*, 341−346.
37. Cariello, N. F., Piegorsch, W. W., Adams, W. T., and Skopek, T. R. (1994) *Carcinogenesis 15*, 2281−2285.
38. Bigger, C. A. H., Pontén, I., Page, J. E., and Dipple, A. (2000) *Mutat. Res. 450*, 75−93.
39. Routledge, M. N. (2000) *Mutat. Res. 450*, 95−105.
40. Canella, K. A., and Seidman, M. M. (2000) *Mutat. Res. 450*, 61−73.
41. Mah, M. C.-M., Boldt, J., Culp, S. J., Maher, V. M., and McCormick, J. J. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 10193−10197.
42. Yang, J.-L., Maher, V. M., and McCormick, J. J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 3787−3791.
43. Boldt, J., Mah, M. C.-M., Wang, Y.-C., Smith, B. A., Beland, F. A., Maher, V. M., and McCormick, J. J. (1991) *Carcinogenesis 12*, 119−126.
44. Bigger, C. A. H., Flickinger, D. J., Strandberg, J., Pataki, J., Harvey, R. G., and Dipple, A. (1990) *Carcinogenesis 11*, 2263−2265.
45. Juedes, M. J., and Wogan, G. N. (1996) *Mutat. Res. 349*, 61−61.
46. Sikpi, M. O., Freedman, M. L., Ziobron, E. R., Upholt, W. B., and Lurie, A. G. (1991) *Int. J. Radiat. Biol. 59*, 1115−1126.
47. Keyse, S. M., Amaudruz, F., and Tyrrell, R. M. (1988) *Mol. Cell. Biol. 8*, 5425−5431.
48. Shibutani, S., and Dasaradhi, L. (1997) *Biochemistry 36*, 13010−13017.
49. Lewis, P. D., Harvey, J. S., Waters, E. M., Skibinski, D. O. F., and Parry, J. M. (2001) *Mutagenesis 16*, 503−515.
50. Courtemanche, C., and Anderson, A. (1999) *Mutat. Res. 430*, 23−36.
51. Maccubin, A. E., Mudipalli, A., Nadadur, S. S., Ersing, N., and Gurtoo, H. L. (1997) *Environ. Mol. Mutagen. 29*, 143−151.
52. Wang, P., Bennett, R. A. O., and Povirk, L. F. (1990) *Cancer Res. 50*, 7527−7531.
53. Jones, R. M., Yuan. Z. X., and Lim, C.-K. (1999) *Rapid Commun. Mass Spectrom. 13*, 211−215.
54. Seidman, M. M., Bredberg, A., Seetharam, S., and Kraemer, K. H. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4944−4948.
55. Chen, R.-H., Maher, V. M., and McCormick, J. J. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8680−8684.
56. Inga, A., Iannone, R., Degan, P., Campomenosi, P., Fronza, G., Abbondandolo, A., and Menichini, P. (1994) *Mutagenesis 9*, 67−72.
57. Shibutani, S., Reardon, J. T., Suzuki, N., and Sancar, A. (2000) *Cancer Res. 60*, 2607−2610.

BI025575I