ABSTRACT
Tamoxifen elevates the risk of endometrial tumours in women and α-(N²-deoxyguanosinyl)-tamoxifen adducts are reportedly present in endometrial tissue of patients undergoing therapy. Given the widespread use of tamoxifen there is considerable interest in elucidating the mechanisms underlying treatment-associated cancer. Using a combined experimental and multivariate statistical approach we have examined the mutagenicity and potential consequences of adduct formation by reactive intermediates in target uterine cells. pSP189 plasmid containing the supF gene was incubated with α-acetoxytamoxifen or 4-hydroxytamoxifen quinone methide (4-OHtamQM) to generate dG-N²-tamoxifen and dG-N²-4-hydroxytamoxifen, respectively. Plasmids were replicated in Ishikawa cells then screened in Escherichia coli. Treatment with both α-acetoxytamoxifen and 4-OHtamQM caused a dose-related increase in adduct levels, resulting in a damage-dependent increase in mutation frequency for α-acetoxytamoxifen; 4-OHtamQM had no apparent effect. Only α-acetoxytamoxifen generated statistically different supF mutation spectra relative to the spontaneous pattern, with most mutations being GC→TA transversions. Application of the LwPy53 algorithm to the α-acetoxytamoxifen spectrum predicted strong GC→TA hotspots at codons 244 and 273. These signature alterations do not correlate with current reports of the mutations observed in endometrial carcinomas from treated women, suggesting that dG-N²-tam adduct formation in the p53 gene is not a prerequisite for endometrial cancer initiation in women.

INTRODUCTION
Tamoxifen is an anti-oestrogen widely used for the treatment, and more recently chemoprevention of breast cancer. The use of tamoxifen in healthy women at a high risk of developing breast cancer is supported by clinical trials demonstrating a 48% reduction in the incidence of oestrogen-receptor-positive breast cancers (1). Although tamoxifen therapy is relatively well tolerated, it is associated with a number of serious side effects, including a 2- to 8-fold increased risk of endometrial cancer (2,3). The underlying mechanisms of tamoxifen carcinogenesis in humans are currently undefined. Long-term administration to rats causes hepatic carcinomas through a genotoxic mechanism involving activation and DNA adduct formation (4) and considerable interest is centred on identifying the role of DNA damage in tamoxifen-induced endometrial carcinogenesis.

In rat liver the primary route of tamoxifen adduct formation involves cytochrome P450 CYP3A4-mediated α-hydroxylation (5) followed by SULT2A1 catalysed α-sulphation to generate a reactive carbocation that binds predominantly to deoxyguanosine, forming α-(N²-deoxyguanosinyl)-tamoxifen (dG-N²-tam) (6,7). α-Hydroxytamoxifen, which can itself react weakly with DNA, is a circulating metabolite in the plasma of women on tamoxifen therapy (8). Furthermore, local activation of both the parent drug and α-hydroxytamoxifen in uterine tissue is a possibility given the presence of measurable CYP3A4 protein and SULT2A1 mRNA expression in this organ (9). More crucially, the potential for tamoxifen adduct formation has been demonstrated in lymphocytes (10), uterine (11–13) and colon tissue (14) of treated women, although evidence to the contrary has also been reported (15,16).

One of the main tamoxifen phase I metabolites, 4-hydroxytamoxifen (4-OHtam), may also be activated to a DNA reactive species, either via α-hydroxylation or...
through formation of a quinone methide (17). Reaction of chemically or enzymatically activated 4-OHtam with deoxyguanosine in vitro produces geometric isomers of 2-(N'-deoxyguanosinyl)-4-hydroxytamoxifen (dG-N2-4-OHtam), a 4-hydroxylated version of the major dG-N2-tam adduct. Whether this adduct is formed in vivo has not been clarified, however, dG-N2-4-OHtam generated by incubation of 4-OHtam with horseradish peroxidase and calf thymus DNA, appears to co-elute with a minor hepatic adduct in tamoxifen-treated rats when compared by HPLC-[32P]-post-labelling analysis (18).

Both dG-N2-tam and dG-N2-4-OHtam adducts are established promutagenic lesions; site-specific studies in vitro (19) or in COS-7 cells (20) have shown that dG-N2-tam primarily directs the misincorporation of DAMP, resulting in GC→TA transversions. Additionally, we have previously reported elevated mutation frequencies when DNA damage generated by 4-OHtamQM and 2-4-OHtam lesions in human uterine cells. The resulting mutagenicity of dG-N2-tam and dG-N2-4-OHtam adducts is replicated in cultured human fibroblasts and kidney cells (21,22). The question of whether tamoxifen adducts are mutagenic in target human endometrial cells has not yet been addressed.

The presence of characteristic mutation spectra in key genes isolated from human tumours has been used as evidence of a causal association between exposure to a certain genotoxic carcinogen, for example UV radiation, aristolochic acid or aflatoxin B1 and cancer initiation (23–25). As such, this approach is one of the most convincing methods available for demonstrating that agent-specific DNA lesions are directly involved in cancer development. The LwPy53 algorithm (26) utilizes experimental data generated using the supF forward mutation assay to predict chemical-induced hotspots along the human p53 gene. The algorithm was recently used to predict the p53 benzo[α]pyrene diolepoxide (BPDE) mutation distribution (26), revealing strong mutation hotspots at codons 157, 248 and 273 that correlate with known preferential sites of BPDE adduct formation within the p53 gene as well as the major mutation hotspots in lung tumours of smokers (27).

In order to advance understanding of the biological significance of tamoxifen DNA adduct formation we have examined the mutagenicity of dG-N2-tam and dG-N2-4-OHtam lesions in human uterine cells. The resulting spectra were compared to the mutation pattern produced by tamoxifen administration in the liver of transgenic rats, to establish the degree of similarity and validate the approach. Subsequently, the supF data were used to predict the mutation distribution in the human p53 gene that might be expected in endometrial tumours of tamoxifen-treated women if DNA adducts play a role in the early stages of cancer development.

**MATERIALS AND METHODS**

**Chemicals and reagents**

The trans-isomers of α-hydroxytamoxifen and α-acetoxytamoxifen (≥99% pure by 1H NMR) were synthesized according to published methods (28). [γ-32P]ATP (>185 TBq/mmol, >5000 Ci/mmol, 370 MBq/ml) was purchased from Amersham, Buckinghamshire, UK. T4 Polynucleotide kinase (3'-phosphatase-free) and calf spleen phosphodiesterase were bought from Boehringer Mannheim, Lewes, UK. All other chemicals were from Sigma, Poole, Dorset, UK, whilst all remaining enzymes and associated incubation buffers were from New England Biolabs Ltd, Hitchin, UK, unless otherwise stated.

**Shuttle vector plasmid, bacterial strain and human cell lines**

The plasmid pSP189 containing the supF gene and Escherichia coli strain MBM7070 (21) were gifts from Dr M. Seidman (National Institute of Aging, NIH, Baltimore, MD, USA). Human endometrial adenocarcinoma (Ishikawa) cells were cultured from cells provided by Dr Ian White (University of Leicester). Ishikawa cells were grown in DMEM/F12, phenol red-free (Invitrogen, Carlsbad, CA, USA) medium supplemented with 10% fetal calf serum (Life Technologies Ltd, Paisley, UK) and 2 mM GlutaMAX at 37°C in 5% CO2 in air.

**In vitro methylation of pSP189 plasmid**

Dried pSP189 plasmid (50 μg) was methylated in vitro using 50 U of CpG methyltransferase (M.SssI) and 32 mM S-adenosylmethionine (5 μl) in NEBuffer 2 (50 mM sodium chloride, 10 mM Tris–HCl, 10 mM magnesium chloride, 1 mM dithiothreitol; pH 7.9 at 25°C), in a total reaction volume of 250 μl. Following a 2 h incubation at 37°C, the enzyme was heat inactivated at 65°C for 20 min and methylated pSP189 plasmid was purified using Sureclean (Bioline, Bath, UK) before reaction with α-acetoxytamoxifen or 4-OHtamQM.

To confirm successful methylation of the plasmid prior to use in adduction reactions, aliquots (2 μg) of methylated or unmethylated (control) pSP189 were digested with 50 U of HpaII in NEBuffer 1 (10 mM bis-Tris–propane-HCl, 10 mM MgCl2, 1 mM dithiothreitol; pH 7.0 at 25°C) in a 60 μl reaction volume for 1 h at 37°C. HpaII does not cleave at restriction sites that have been CpG methylated by M.SssI but will digest control pSP189 plasmid within 5'-CCGG recognition sites. Methylation was therefore confirmed by comparing the reaction products on a 1% agarose gel (data not shown).

**Treatment of pSP189 plasmid with α-acetoxytamoxifen**

Aliquots of methylated pSP189 plasmid (200 μg in 200 μl Tris–EDTA buffer, pH 8.0) were treated with varying doses of α-acetoxytamoxifen dissolved in 100 μl 50:50 ethanol:acetonitrile (10, 25 and 50 μM final concentrations) and the total volume adjusted to 500 μl with water. Samples were prepared in duplicate, along with a single solvent [ethanol:acetonitrile (50:50)] treated control and incubated overnight at 37°C. Unreacted compound and degradation products were then extracted with water saturated ethyl acetate (4 x 400 μl) and the DNA precipitated by addition of 3 M sodium acetate (10 μl) and ice-cold ethanol (800 μl). The DNA samples were pelleted by centrifugation and washed with 70% ice-cold ethanol, followed by 100% ice-cold ethanol. Pellets were then re-dissolved in 200 μl sterile tissue culture grade water.
Treatment of pSP189 plasmid with 4-OHtamQM

4-Hydroxytamoxifen was activated to its quinone methide as previously described (21). 4-OHtamQM (in 50 : 50 ethanol : acetonitrile) was added to 200 µg methylated pSP189 plasmid (in 400 µl H2O) in a total volume of 500 µl, to produce final concentrations of 0, 50, 100 and 250 µM. After overnight incubation at 37°C, unreacted 4-OHtamQM was extracted using water saturated diethyl ether (5 × 400 µl). Plasmid DNA was then precipitated and re-dissolved as detailed above.

HPLC-32P-post-labelling analysis of adducted pSP189 plasmid DNA

To quantify the number of tamoxifen-DNA adducts formed on the treated plasmids, aliquots of DNA (10 µg) were analysed using our established 32P-post-labelling assay, with HPLC separation of 32P-post-labelled nucleotides and online radiochemical detection (18). A positive control sample, liver DNA from a tamoxifen treated rat, was analysed in parallel to enable adduct identification (18,29).

Transfection and transformation

Subconfluent Ishikawa cells were transfected with α-acetoxytamoxifen or 4-OHtamQM treated plasmid (10 µg per 9-cm culture plate) using the Fugene-6 (Roche Diagnostics Ltd, Lewes, UK) transfection reagent (30). The plasmid was reclaimed from the Ishikawa cells between 45 and 48 h after transfection using plasmid purification kits (Qiagen, Crawley, UK). Aliquots were used to transform electrocompetent MBM7070 E. coli (25). After overnight incubation at 37°C, unreacted 4-OHtamQM was extracted using water saturated diethyl ether (50 : 50 ether (50–250 µl). Plasmid DNA was then precipitated and re-dissolved as detailed above.

RESULTS

Tamoxifen-DNA adduct quantification by 32P-post-labelling

The shuttle vector plasmid pSP189 was modified by reaction with α-acetoxytamoxifen or 4-OHtamQM and aliquots were subjected to HPLC-32P-post-labelling analysis to quantify the adduct levels formed. The concentrations used were chosen on the basis of our previous studies in Ad293 kidney cells (21), which found that treatments with α-acetoxytamoxifen in the range 10–50 µM were required to generate significantly mutagenic levels of damage, whilst higher concentrations of 4-OHtamQM (50–250 µM) were necessary due to this derivative forming lower adduct numbers per molar equivalent. The adduct profiles induced by the two compounds were comparable with our previous report in which the pSP189 plasmid employed was used directly after isolation from E. coli (21) and therefore contained the Dam methylation pattern rather than mammalian CpG methylation. The rationale...
underlying the requirement for plasmid methylation is based on the fact the p53 gene is methylated in a variety of human tissues and many chemical carcinogens exhibit a preference for binding at methylated CpG sites. Furthermore, the LwPy53 algorithm has been shown capable of predicting the p53 mutation spectrum in lung cancers from smokers using BPDE derived supF data in methylated plasmid, whereas there was no such correlation when unmethylated plasmid was used (26). The present findings indicate that the presence of CpG methylation does not overtly affect the types and proportions of specific adducts formed (18,21). Treatment of plasmid with α-acetoxytamoxifen resulted in the formation of one major peak (Figure 1B) consistent with the dG-N2-tam adduct detected in hepatic DNA from a tamoxifen-treated rat (Figure 1D). An additional minor peak is also observed eluting just prior to the major adduct, which has previously been suggested to be a diastereoisomer of trans-dG-N2-tamoxifen (29).

Following incubation of 4-OHtamQM with plasmid DNA, two main 32P-post-labelled adduct peaks are detected, presumably cis- and trans-isomers of dG-N2-4-OHtam (Figure 1C) (17). Both of these adducts have similar chromatographic properties to minor DNA adduct peaks found in the rat liver tissue (Figure 1D).

As expected, for both reactive derivatives adduct levels increased with increasing concentration in a dose-dependent manner (Figure 2A). For the same exposure (50 µM) 4-OHtamQM induced a 95-fold lower level of tamoxifen DNA adducts per plasmid than α-acetoxytamoxifen. Based on a plasmid size of 4952 bp the degree of pSP189 modification by α-acetoxytamoxifen equates to a level of 1.8, 3.0 or 3.8 adducts per plasmid for the 10, 25 and 50 µM doses, respectively. In comparison, the three treatments with 4-OHtamQM (50, 100 and 250 µM) induced ~0.04, 0.08 and 0.31 adducts per plasmid, damage levels that have previously proven highly mutagenic.

![Figure 1](image_url). Representative HPLC chromatograms of 32P-post-labelled DNA digests from untreated solvent control plasmid (A) or plasmid samples incubated with α-acetoxytamoxifen (B) or 4-hydroxytamoxifen quinone methide (C), and liver DNA from a tamoxifen-treated Wistar Han rat (40 mg/kg tamoxifen in the diet for 6 months) (D). The amount of DNA injected was 9.1 µg for all samples except the α-acetoxytamoxifen adducted plasmid (0.5 µg).
Mutation frequency in \textit{supF} gene

As illustrated in Figure 2B, upon replication in Ishikawa cells \(\alpha\)-acetoxytamoxifen-induced DNA adducts caused a dose-dependent increase in mutation frequency relative to the solvent control (3.1-fold higher for 10 \(\mu\)M, 10.5-fold for 25 \(\mu\)M and 18.4-fold for 50 \(\mu\)M). In contrast, no such dose or damage-related effect was apparent with 4-OHtamQM-treated plasmid. In this case, the highest mutation frequency was evident in plasmid treated with 100 \(\mu\)M 4-OHtamQM (9.6\(\times10^8\) colonies); however, this was only 1.96-fold higher than the spontaneous mutation frequency in the solvent control plasmid and equates to a weak, if any, mutagenic effect. Overall, \(\alpha\)-acetoxytamoxifen-treated plasmid generated 631 mutant colonies out of a total of 246,494, whilst 4-OHtamQM produced 290 mutants out of 375,301 colonies. Similar numbers of mutant colonies for each treatment (\(\approx 200\)) were then sequenced to generate mutation spectra.

Mutation type in the \textit{supF} gene

White mutant colonies were collected and the \textit{supF} gene sequenced to identify the types and location of mutations. The majority of mutations resulting from replication of both \(\alpha\)-acetoxytamoxifen and 4-OHtamQM-induced damage were base substitutions (79 and 63\% of all mutants, respectively). For \(\alpha\)-acetoxytamoxifen, most of these were in the form of single base alterations (90\%), with a few tandem (2\%) and a larger proportion of multiple substitutions (8\%; two or more substitutions at non-adjacent sites along the \textit{supF} gene). In mutant colonies derived from 4-OHtamQM-treated plasmid, single substitutions were again predominant (78\% of all substitutions), followed by multiple (16\%) and tandem (6\%) substitutions.

Small frameshifts were a relatively infrequent consequence of both \(\alpha\)-acetoxytamoxifen and 4-OHtamQM-induced damage, detected in just 9 and 3\% of mutant plasmids, respectively. When present, the majority of frameshifts in \(\alpha\)-acetoxytamoxifen-treated plasmid took the form of single base deletions accounting for 68\% of the total, while single base insertions were more common (67\% of all frameshifts) in 4-OHtamQM-treated plasmid. Interestingly, a large deletion of bases 97\(\rightarrow\)267 (including the whole \textit{supF} gene) was present in 40\% of the combined control mutants sequenced, 12\% of \(\alpha\)-acetoxytamoxifen and 33\% of 4-OHtamQM-treated plasmids. The occurrence of this large 171-bp deletion decreased markedly upon exposure to \(\alpha\)-acetoxytamoxifen, accounting for 29, 9 and 1\% of all mutations in the 10, 25 and 50-\(\mu\)M-treated plasmids respectively, as the profile is less influenced by spontaneous alterations and becomes more characteristic of \(\alpha\)-acetoxytamoxifen. The nature of this background mutation is uncertain, although there is strong evidence it is related to short direct repeats that mediate deletions and duplications in DNA (39,40). Two possible mechanisms for these events are recombination between short homologous repeats and DNA polymerase slippage between short repeated sequences. Short-homology-independent illegitimate recombination (41) requires short regions of homology between recombination sites such as the 5-bp sequence (GAGCT) in the junction of the 171-bp deletion observed in the present study.

Figure 3 illustrates the effect of increasing dose and adduct level on the single base substitution profiles induced by \(\alpha\)-acetoxytamoxifen and 4-OHtamQM. Each type of substitution is expressed as a percentage of the total substitutions detected for each dose. For \(\alpha\)-acetoxytamoxifen-modified plasmid (Figure 3A), the increasing dose is mirrored by an increase in the proportion of GC\(\rightarrow\)TA transversions, which become the predominant substitution beyond 10\(\mu\)M. At the same time, the proportion of GC\(\rightarrow\)AT transitions, the alteration that dominates the spontaneous spectrum, decreases with dose. The mutation pattern produced by replication of \(\alpha\)-acetoxytamoxifen adducted plasmid in human endometrial cells is consistent with that resulting from replication.

**Figure 2.** Dose–response relationships for DNA adduct formation and mutation induction. DNA adduct levels in plasmid treated with \(\alpha\)-acetoxytamoxifen (dashed line) and 4-OHtamQM (solid line) determined by HPLC-\(^{32}\)P-post-labelling analysis (A). Data represent the mean \(\pm\) SD of duplicate measurements of two plasmid samples per dose. For each chart the 0\(\mu\)M dose represents control pSP189 plasmid incubated with solvent only. The lower chart illustrates the relationship between mutation frequency and DNA adduct level for damage induced by \(\alpha\)-acetoxytamoxifen and 4-OHtamQM, with the insert corresponding to a magnification of the 4-OHtamQM data (B). The mutation frequency of control plasmid suspended in water only was 7.2\(\times10^5\) colonies, which is comparable to that observed for plasmid incubated with solvent, 4.9\(\times10^5\) colonies. The presence of ethanol:acetonitrile (50:50) was therefore adjudged to have no appreciable effect on spontaneous mutagenesis and data from both types of control were combined to increase the pool of mutated colonies for subsequent sequence analysis.
of dG-N²-tam in several other cellular systems and can be considered characteristic of the damage present (20–22). In contrast, there is little obvious difference between the various 4-OHtamQM treatments, with the most frequent single base substitution being GC→AT transitions at all concentrations. Furthermore, the overall substitution profile for 4-OHtamQM adducted plasmid was virtually indistinguishable from that observed in spontaneous mutants derived from untreated control plasmid (Figure 3B).

Analysis of mutation spectra in the supF and human p53 genes
The distribution of single base and tandem substitution mutations within the supF gene for plasmids treated with either α-acetoxytamoxifen (25 and 50 µM combined) or 4-OHtamQM (50, 100 and 250 µM combined) is shown in Figure 4. When these spectra are compared to the untreated control spectrum, which is comprised of spontaneous mutations using the Adams–Skopek hypergeometric test (42), only the α-acetoxytamoxifen spectrum is found to be significantly different (P = 0.00006). Within the mutation spectrum induced in control plasmid there are three hotspots at positions 129, 133 and 139. In the α-acetoxytamoxifen combined spectrum six hotspots are evident at positions 115, 129, 159, 160 and 168, all of which are at GC sites, consistent with guanine being the primary site of tamoxifen adduction. Five hotspots are apparent in the spectrum of single base substitutions induced by 4-OHtamQM, at positions 108, 129, 133, 139 and 168, all at sites of GC basepairs. Only one hotspot at position 129 was shared between the α-acetoxytamoxifen and control spectra, whereas all three control hotspots (at positions 129, 133 and 139) also appear in the 4-OHtamQM spectrum. One further hotspot (position 168) was common to both the α-acetoxytamoxifen and 4-OHtamQM spectra.

Only α-acetoxytamoxifen exposure resulted in an increased mutation frequency and a spectrum significantly different from the spontaneous pattern. Therefore, these data alone (25 and 50 µM doses combined) were used for subsequent analyses. Using HCA comparisons can be made between multiple carcinogen-induced mutation spectra produced in different experimental systems, regardless of sequence (33). Adopting this approach the pattern and type of mutations observed in the present study with α-acetoxytamoxifen were compared with base substitutions generated in the cII gene isolated from liver tissue of tamoxifen and α-hydroxytamoxifen-treated Big Blue transgenic rats (37, 38). In addition, comparisons were made with mutation spectra induced by exposure to UVB, UVC, simulated sunlight, BPDE or hydroxy radicals in the cII, supF and yeast p53 model systems (30–36). Results revealed a high degree of similarity between the three tamoxifen spectra, which all grouped closely together forming a separate subcluster (Figure 5). Furthermore, the tamoxifen mutational signature was distinct from that caused by the other mutagens, which also clustered according to the specific chemical or exposure, independently of the assay system employed.

The LwPy53 algorithm was then applied to the α-acetoxytamoxifen supF data to predict the distribution of mutations that might be expected to arise in the human p53 gene as a consequence of dG-N²-tam adduct formation in endometrial tissue. Hypothetical treatment with α-acetoxytamoxifen produced two GC→TA hotspots in the human p53 gene at codons 244 and 273 of exons 7 and 8, respectively (Figure 6).

DISCUSSION
Evidence suggests tamoxifen is capable of forming DNA adducts in the endometrial tissue of treated women, albeit at low levels and with a degree of inter-individual variability. Since little is understood about the actual role of tamoxifen adduct formation in endometrial cancer development, the primary aim of this work was to investigate the mutagenicity and potential biological consequences of tamoxifen adducts in this target tissue. The supF gene has been used as target for mutagenesis by a wide variety of mutagens but virtually all of the work to date has been performed using non-CpG methylated plasmid. By methylating the pSP189 plasmid in vitro prior to tamoxifen adduction we were able to exploit the LwPy53 algorithm.
**α-Acetoxytamoxifen**

| Combined Control | Treated |
|------------------|---------|
| AAAA             | TTA     |
| A                | TA      |
| C                | GA      |
| G                | A       |
| T                | A       |
| G                | A       |
| A                | T       |
| T                | A       |
| G                | TG      |
| A                | TG      |
| A                | TAT     |
| T                | ATT     |
| A                | TAT     |
| C                | TT      |
| A                | TAT     |
| T                | AT      |
| G                | TG      |
| A                | GA      |
| T                | AT      |
| T                | A       |
| C                | AA      |
| A                | A*      |
| A                | A       |
| GA               | C       |
| G                | A       |
| A                | T       |
| T                | A       |
| G                | AT      |
| T                | AT      |
| A                | T       |
| T                | A       |
| C                | AA      |
| A                | A       |
| A                | A       |
| GG               | AT      |
| T                | AT      |
| A                | TA      |
| C                | TT      |
| A                | TAT     |
| A                | TAT     |
| C                | TT      |
| T                | TAT     |
| A                | TAT     |
| C                | AA      |
| A                | A*      |
| A                | A       |
| GA               | C       |
| G                | A       |
| A                | T       |
| T                | A       |
| G                | AT      |
| T                | AT      |
| A                | T       |
| T                | A       |
| C                | AA      |
| A                | A       |
| A                | A       |

**4-OHTamQM**

| Combined Control | Treated |
|------------------|---------|
| AAAA             | TTA     |
| A                | GA      |
| A                | A       |
| A                | A       |
| A                | A       |
| A                | G       |
| G                | A       |
| A                | G       |
| G                | T       |
| A                | T       |
| T                | A       |
| G                | TG      |
| A                | GA      |
| T                | AT      |
| T                | AT      |
| A                | T       |
| T                | A       |
| G                | TG      |
| A                | GA      |
| T                | AT      |
| T                | A       |
| C                | AA      |
| A                | A       |
| A                | A       |
| G                | A       |
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| A                | A       |

Figure 4. Mutation spectra depicting single base and tandem substitutions in the supF gene induced in human endometrial cells by α-acetoxytamoxifen (25 and 50 μM) and 4-hydroxytamoxifen quinone methide (50, 100 and 250 μM). The 5–3' 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 showing the position and type of point mutations induced in the combined control sample. Hotspots (represented by asterisks) are assumed when the number of mutations observed is 4-fold or more greater than the number expected for a random (Poisson) distribution (21,22). Tandem mutations are underlined.
for the prediction of GC→TA transversion hotspots along exons 5, 7 and 8 of the human p53 gene in response to α-acetoxytamoxifen treatment.

CpG methylation of the pSP189 plasmid DNA prior to incubation with the model reactive metabolites α-acetoxytamoxifen and 4-OHtamQM had no obvious effect on gross adduct number or the general profile of damage induced, as assessed by HPLC-32P-post-labelling. Tamoxifen adducts were formed in a dose-dependent manner, with levels ranging from 175 to 376 and 4 to 30 adducts/10⁶ nt for α-acetoxytamoxifen and 4-OHtamQM, respectively, which are similar to that produced in non-methylated plasmid (50–240 and 5–20/10 ⁶ nt) using identical concentrations in our previous studies (21,22). It is however quite feasible that although the degree of adduction was unaffected, positions of adduct formation along the supF gene or lesion conformation may be influenced by CpG methylation and this alone may be sufficient to significantly alter mutagenic outcome (43,44).

Replication of α-acetoxytamoxifen-derived damage in human endometrial cells resulted in a dose-related increase in mutation frequency (Figure 2B). This elevation was apparent from the lowest dose (10 μM), where the relative mutation frequency was treble the spontaneous rate in solvent treated plasmid and it reached a maximum of 18.4-fold above background at the 50 μM concentration. The mutagenic potency of α-acetoxytamoxifen-derived damage in endometrial cells is greater than in both human kidney cells and fibroblasts, when compared in terms of relative mutation frequency for a given adduct level.

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**Figure 5.** HCA dendrogram to reveal similarities between transformed mutation signatures. The UV-induced mutation signatures group separately to form a cluster containing the remaining signatures due to a predominance of the GC→AT mutation type. However, in the second cluster the three tamoxifen signatures in the supF and cII genes form their own, distinct subcluster. This subcluster demonstrates that the tamoxifen spectra are very similar in sequence context and mutation type (A). Table describing details of the mutation spectra used (B).

| Code  | Mutagen    | Gene | Tissue origin/Test system | Reference |
|-------|------------|------|----------------------------|-----------|
| UBs   | UVB        | supF | XP-A fibroblast/supF assay | 34        |
| UCy2  | UVC        | p53  | Yeast p53 functional assay | 35        |
| SUNc  | Simulated sunlight | cII | Fibroblast/Big Blue mouse | 32        |
| UCs   | UVC        | supF | Human kidney/supF assay    | 33        |
| UCy1  | UVC        | p53  | Yeast p53 functional assay | 36        |
| UBc   | UVB        | cII  | Embryonic fibroblasts/Big Blue mouse | 31 |
| UCc   | UVC        | cII  | Fibroblast/Big Blue mouse  | 32        |
| Tc    | α-Hydroxytamoxifen | cII | Liver/Big Blue rat | 38        |
| Ts    | α-Acetoxytamoxifen | supF | Human endometrium/supF assay | Present study |
| Tc2   | Tamoxifen  | cII  | Liver/Big Blue rat        | 37        |
| BPy2  | Benzo[a]pyrene | p53 | Yeast p53 functional assay | 35        |
| BPc   | Benzo[a]pyrene | cII | Embryo fibroblast/Big Blue mouse | 30        |
| OH    | Hydroxyl radical | p53 | Yeast p53 functional assay | 33        |
| BPy1  | Benzo[a]pyrene | p53 | Yeast p53 functional assay | 33        |
| BPs   | Benzo[a]pyrene | supF | XP-A fibroblast/supF assay | 30        |
Replication of just 3.0 lesions per plasmid caused a 10.5-fold increase in mutation frequency in endometrial cells, whilst 2.5 and 3.4 adducts per plasmid resulted in a 5-and 1.2-fold increase in kidney and fibroblast cells respectively (21,22). Comparison of the 3-acetoxytamoxifen induced supF mutation spectrum (Figure 4) with results obtained in kidney cells and repair proficient fibroblasts (21,22) using the Adams–Skopek test revealed both published spectra, which were derived from unmethylated plasmid, to be significantly different from that produced in endometrial cells. Therefore, whilst it is possible that CpG methylation of the plasmid used in the present study is contributing to the enhanced effect, the results suggest that the major dG-N²-tamoxifen adduct, which is already considered to be a potent mutagenic lesion (21), poses an even higher mutation risk in endometrial cells. Previously published supF studies with bulky carcinogens have reported lower levels of mutagenicity. Specifically, when compared on an adduct per plasmid basis, 3-acetoxytamoxifen is about 39 times more mutagenic than benzo[c]-phenanthrene (45), 22-fold more mutagenic than 1,6-dinitropyrene (46) and 9–18 times more mutagenic than BPDE-derived damage (47,48).

The prevalent spontaneous substitution mutation in control plasmids were GC→AT transitions, consistent with previously reported supF mutation spectra in human Ad293 cells (49), lymphoblasts (50) and monkey kidney cells (51). The introduction of increasing levels of 3-acetoxytamoxifen-induced damage causes a clear dose-dependent shift towards GC→TA transversions, which become the predominant mutation beyond 10 μM (Figure 3A) and the proportion of total mutations caused by spontaneous events decreases. Lower, but significant levels of GC→AT transitions are also still observed at the higher doses. Site-specific mutation studies in COS-7 cells (20), previous supF experiments (21,22), primer extension reactions in vitro (19,52) and in vivo studies with tamoxifen treated transgenic rats (53) have established GC→TA transversion mutations as the primary misincorporation product of translesion synthesis past dG-N²-TAM adducts, with GC→AT transitions also apparent under certain circumstances. The present results therefore confirm GC→AT mutations as being characteristic of damage induced by 3-acetoxytamoxifen and by inference, 3-hydroxytamoxifen and 3-sulphotetamoxifen (if formed), in human endometrial cells.

Comparison of mutation spectra constructed from sequence analysis of spontaneous and treatment-induced mutant plasmids using the Adams–Skopek test, revealed that the single and tandem base substitution spectra from the two top 3-acetoxytamoxifen doses (25 and 50 μM) were statistically different from the combined control (P < 0.05). These were therefore combined into a single spectrum with a total of six hotspots evident, at positions 115, 123, 129, 159, 160 and 168. Hotspots at base pairs numbered 159 and 160 were also observed in the mutation spectrum arising from replication of 3-acetoxytamoxifen induced damage in Ad293 kidney cells (21), whilst those at positions 115, 123 and 168 also seem to be characteristic of this treatment by virtue of the fact they were not detected in the control spectra. Position 129 was a hotspot in all 3-acetoxytamoxifen and untreated control spectra, except for the top 3-acetoxytamoxifen dose (50 μM). A single hotspot at the same position (129) was the only one observed in a previously reported supF spontaneous spectrum generated in Ad293 cells, suggesting alterations here may be induced by 3-acetoxytamoxifen or could be due to background endogenous damage (49). Two other hotspots (133 and 139) were present in the combined control spectrum and were not observed in any 3-acetoxytamoxifen induced spectra, implying these mutations arose purely through spontaneous means. This conclusion is further supported by a recent comparative analysis of mammalian cell line spontaneous mutation spectra in supF (54), in which hotspot C133 was the most frequent and hotspots at G129 and C139 were also common.

It is interesting that compared to the clear mutagenicity of 3-acetoxytamoxifen-derived adducts, treatment of methylated pSP189 with 4-OHtamQM failed to convincingly raise the mutation frequency above background control levels; absolute mutation frequencies were only slightly higher than the spontaneous value of 4.9/10⁴ colonies and were unrelated to dose. This finding is in sharp contrast to the mutation frequencies reported in our analogous supF investigation of 4-OHtamQM in Ad293 cells (21), which revealed 4-OHtamQM induced damage to be ~25 times more mutagenic than 3-acetoxytamoxifen-derived adducts on an adduct per plasmid basis. Although the reason(s) behind the specific lack of mutagenicity in endometrial cells is unclear, there are several possible explanations. Importantly, identical protocols for the synthesis of 4-OHtamQM and plasmid adduction were adopted in each study and the levels of adducts detectable by ³²P-post-labelling were remarkably similar, which indicates the difference in mutagenicity cannot be attributed to variations in the extent of dG-N²-4-OHtam adduct formation. However, the use of Cpg methylated plasmid in the present study could influence the overall damage profile. Whilst there was no obvious difference in the adduct pattern detected by ³²P-post-labelling, we have previously ascribed the induction of relatively high numbers of
multiple mutations and large deletions in 4-OHTamQM-treated plasmid to the presence of dimer adduct cross-links or free radical induced strand breaks, both of which would probably be undetectable by the $^{32}$P-post-labelling assay employed (21). DNA methylation at CpG sites could potentially reduce the likelihood of dimer formation and may also alter the conformational and/or miscoding properties of any resulting dG-N$^\prime$-4-OHTam adducts or cross-links, rendering the damage less mutagenic. Finally, cell specific factors (e.g. different polymerase profiles) could contribute to, or even account entirely for, the different mutagenic processing of 4-OHTamQM-derived lesions in endometrial cells (55).

Consistent with the lack of mutagenic activity, the profile of single-tandem and multiple substitutions generated by 4-OHTamQM was similar to the types of spontaneous mutations observed in the control plasmid (Figure 3B). Furthermore, the 4-OHTamQM mutation spectra were statistically similar to the untreated control spectra ($P > 0.05$), sharing three hotspots (at positions 129, 133 and 139) in the combined single-tandem spectrum (Figure 4). Taken together, the evidence suggests that replication of 4-OHTamQM-induced damage in human endometrial cells has no real effect on the background spontaneous mutagenicity and implies any compound specific lesions generated by 4-hydroxytamoxifen do not play a major role in tamoxifen-induced cancers.

Recently, we have described a novel strategy for comparing mutation spectra in a variety of genes generated in different in vitro/in vivo assay systems, which creates mutational signatures by capturing mutation type and sequence context information simultaneously (33). This methodology was exploited to assess the validity of using tamoxifen supF data to predict in vivo spectra. Interestingly, HCA revealed a strong similarity between mutational signatures for tamoxifen in the supF and cII genes, clearly distinct from that produced by other genotoxic agents BPDE, specific types of UV radiation and hydroxyl radicals. Importantly, these findings strongly support the use of in vitro supF data for the prediction of in vivo mutation spectra in situations where tamoxifen acts as a genotoxic carcinogen, as is the case in rat liver. Furthermore, the results reinforce the theory that if tamoxifen adduct formation in the p53 gene is a factor in endometrial cancer development, a characteristic pattern of mutations, distinguishable from that induced by other DNA adducts, might be expected.

Missense mutations in the gene sequence encoding the DNA-binding domain (exons 5–8) of p53 are found in ~50% of human cancers (56). These alterations result in nuclear accumulation of p53 protein with loss of normal functions. Mutations in the p53 gene are an important early event in the initiation of many types of cancer, meaning that for some tumours arising through genotoxic mechanisms, signature patterns of p53 mutations may be matched aetiologically with exposure to specific carcinogens (57). The majority (>80%) of sporadic endometrial carcinomas is classified as oestrogen-driven, Type I tumours with a good overall prognosis which are characterized by mutations in PTEN, PIK3CA, β-catenin and K-ras, but not in p53. In comparison, the less frequent Type II non-oestrogen-related carcinomas are high-grade with a worse prognosis and exhibit chromosomal instability and p53 mutations (58). There is mounting evidence to suggest that tamoxifen exposure increases the risk of developing type II tumours with unfavourable histological features. These more aggressive tumours seem to occur in women diagnosed years after discontinuation of tamoxifen therapy, whilst Type I tumours are more likely to develop in women during treatment (59). This is consistent with two distinct mechanisms: the oestrogen agonist activity of tamoxifen accounting for Type I tumours whilst a genotoxic hormone independent mechanism involving DNA adduct formation could contribute to the development of Type II tumours (59,60).

Based on the p53 GC→TA transversion predictions, treatment with α-acetoxytamoxifen generates two hotspots at positions 244 and 273. Position 273 in the DNA-binding region of p53 appears to be a common hotspot for bulky adducts, such as those formed by benzo[a]-pyrene, a component of tobacco smoke (61). Overall, codon 273 in exon 8 is one of the most frequently mutated sites in human tumours and resides within regions of slow DNA repair (62). Alteration of this important amino acid residue, which forms contacts between the protein structure and DNA, has a detrimental effect on the ability of p53 to act as a transcription factor in the activation of downstream genes. The predominant mutations occurring at this site in human tumours are GC→AT transitions. However, as is predicted for tamoxifen in the present study, benzo[a]-pyrene exposure induces characteristic GC→TA transversions at codon 273, which enables tobacco-related cancers to be distinguished from spontaneous lung tumours (63). Codon 244 of exon 7 is far less frequently mutated in human cancers, therefore it could potentially provide a specific signature mutation for tamoxifen-induced endometrial carcinomas.

There is very limited published data on the p53 mutation distribution in endometrial tumours occurring in women undergoing tamoxifen therapy and in carcinomas arising spontaneously. Very recently Fujiwara et al. (64) reported the presence of characteristically unique p53 mutation spectra in endometrial carcinomas from tamoxifen-treated women. Of the 23 samples screened, six (26%) contained p53 mutations; four substitutions in exons 5 and 6 (two GC→AT at non-CpG sites, one AT→CG, one AT→GC) and two insertions (both TGT→TGTT at codon 238) were detected. This profile differed considerably from the pattern previously described in sporadic endometrial carcinoma by the same group, where GC→AT mutations were present in 45% of cases, all at methylatable CpG sites (65). Although the number of mutations observed are low, the fact that none occurred at codons 244 or 273, or corresponded to the expected GC→TA alterations preferentially induced by dG-N$^\prime$-tam, suggests that tamoxifen adducts are not directly involved in the generation of p53 mutations in treated women. It is recognized however, that for a more definitive conclusion many more tamoxifen-associated endometrial tumours need to be screened for mutations. The individual spectra generated in this study, and those used by Lewis et al. (33) for other genotoxic agents such as BPDE, each contain at
least 20 mutable sites with over 70 mutations distributed across these sites. This means that the estimated power for any pairwise comparisons of these spectra would be high (66). For example, a test where both spectra contributed a total of 150 mutations across 20 mutable sites would have an estimated power of ~84%. It is currently not appropriate to make such comparisons with the p53 spectrum in endometrial carcinomas from tamoxifen-treated women described by Fujiwara et al. (64) as statistical power would be lacking; the in vivo data would contribute only six mutations, giving a relatively low overall mutation count and power estimated to be considerably <50%.

By employing a combined experimental and multivariate statistical approach, further insight into the role of tamoxifen-induced DNA damage in endometrial carcinogenesis has been gained. The results support the use of supF data for predicting in vivo mutation spectra. Therefore, considering the lack of correlation between the hypothetical p53 mutation spectrum and alterations reported to date in endometrial tumours, it seems that although they may be characteristic of treatment, the mutations are unlikely to be caused by misincorporation opposite dG-N2-tam lesions. Accordingly, based on current evidence dG-N2-tam adduct formation, at least in the p53 gene, does not appear to be a prerequisite for endometrial cancer initiation in women.

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