Investigating DNA Adduct-Targeted Mutagenicity of Tamoxifen: Preferential Formation of Tamoxifen−DNA Adducts in the Human *p53* Gene in SV40 Immortalized Hepatocytes but Not Endometrial Carcinoma Cells[†]

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Received February 28, 2005; Revised Manuscript Received April 10, 2005

ABSTRACT: Tamoxifen is a widely used drug for chemotherapy and chemoprevention of breast cancer worldwide. Tamoxifen therapy is, however, associated with an increased incidence of endometrial cancer. The carcinogenicity of tamoxifen is ascribed to its genotoxic and estrogen agonist effects. We investigated DNA adduct-targeted mutagenicity of tamoxifen as a function of its genotoxicity in the *cII* transgene in Big Blue mouse embryonic fibroblasts and mapped the formation of tamoxifen-induced DNA adducts in the *p53* tumor suppressor gene in SV40 immortalized human hepatocytes and human endometrial carcinoma cells. We used the terminal transferase-dependent polymerase chain reaction for mapping of DNA adducts in the *cII* and *p53* genes. We utilized a λ phage-based assay and DNA sequencing for determining *cII* mutant frequency and mutation spectrum, respectively. Tamoxifen treatment yielded polymerase-blocking DNA adducts at multiple nucleotide positions along the *cII* transgene. The treatment significantly and dose-dependently increased the *cII* mutant frequency ($p < 0.01$), leaving a unique mutation spectrum ($p < 0.0001$) and a signature mutation of G:C → T:A transversions ($p < 0.03$), relative to the control. Tamoxifen treatment of the immortalized human hepatocytes but not endometrial carcinoma cells, even in the presence of an external activation system, i.e., rat liver S9 mix, induced DNA adducts at specific codons along exons 6 and 8 of the *p53* gene. These data suggest a proficient metabolic activation of tamoxifen in human liver and an inefficient activation and/or efficient detoxification of tamoxifen in human endometrium. Because the liver is essentially a mitotically quiescent organ, tamoxifen−DNA adduction in the liver may, at least partially, prevent its reactants from reaching highly proliferative organs via, e.g., circulating blood. Thus, tamoxifen−DNA adduction in the liver may not have as significant biological consequences as it might have in highly proliferative organs. Our findings favor an involvement of a nongenotoxic mechanism in tamoxifen-associated human endometrial cancer.

Tamoxifen is a triphenylethylene nonsteroidal antiestrogen used worldwide as an adjuvant chemotherapeutic agent in the treatment of all stages of breast cancer and as a chemopreventive drug in women at high risk of developing breast cancer. Tamoxifen therapy is, however, associated with an increased risk of endometrial cancer. The underlying mechanism of carcinogenicity for tamoxifen remains elusive although both genotoxic and estrogen agonist properties of tamoxifen have been implicated in the carcinogenic process (reviewed in refs *1*−*3*). In rats, tamoxifen is a hepatocellular carcinogen whose tumorigenic activity correlates with the extent of its DNA binding and mutagenicity (*4*−*8*). In mice, however, tamoxifen does not cause liver cancer, which is consistent with its attenuated DNA-binding ability in this species (*9*, *10*). Despite the nontumorigenicity of tamoxifen in the reproductive organs of adult rodents, there is an elevated incidence of uterine adenocarcinoma in mice transplacentally exposed to this agent (*11*). In addition, neonatal exposure to tamoxifen in both mice and rats results in uterine cancer (*11*, *12*). The latter is ascribed to a partial estrogenic effect of tamoxifen that might cause hormonal perturbation of the developing organs including the uterus (*1*−*3*).

To exert a genotoxic effect, tamoxifen requires metabolic activation to form reactants capable of binding covalently to DNA and yielding complexes of "DNA adducts". Formation of DNA adducts is a key event in the multi-stage process of carcinogenesis (*13*). Unrepaired or inefficiently repaired DNA adducts may cause mispairing during DNA replication, thereby leading to mutations. Specific mutations in crucial genes encoding proteins for cell-cycle control and growth might trigger tumorigenesis (*14*, *15*). *In vivo*, tamoxifen is biotransformed into a variety of metabolites by a battery of phase I activating enzymes. The main derivatives are α-hydroxy-tamoxifen, 4-hydroxy-tamoxifen, *N*-desmethyl-tamoxifen and tamoxifen *N*-oxide. Of these, α-hydroxy-tamoxifen is considered as the proximate carcinogen that further undergoes *O*-sulfation to generate reactive esters. A subsequent loss of sulfate from these esters results in allylic carbocations that are highly DNA reactive. A major reaction

[†] This work was supported by a grant from the National Cancer Institute (CA84469) to G.P.P.

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10.1021/bi0503753 CCC: $30.25
Published on Web 05/17/2005

product is a miscoding DNA adduct, α-(N^2-deoxyguanosinyl)-tamoxifen, in which the α carbon of tamoxifen is covalently bound to the exocyclic N^2 amino group of guanine in four trans/cis diastereoisomers. All isomers are poorly to moderately subject to DNA repair, thus being variably mutagenic. Conjugation of tamoxifen metabolites, however, occurs principally through glucuronidation and biliary excretion, which constitutes a major detoxification pathway (reviewed in refs *1*−*3*).

In humans, tamoxifen genotoxicity as a function of carcinogenicity has been investigated by dosimetry of DNA adducts in the target organ, i.e., endometrium, and in surrogate organs, e.g., leukocytes and liver. So far, however, no consensus has been reached on a genotoxic mechanism contributing to the cancer-causing effects of tamoxifen (*1*, *2*). This ambiguity is to be expected in view of the methodological differences in quantifying tamoxifen−DNA adducts in various studies. The widely utilized ^{32}P-postlabeling-based assays have used dissimilar digestion of DNA and variable adduct enrichment and chromatographic procedures in different studies. Additionally, incomparable phosphorylation efficiencies as well as the use (versus not using) of authentic DNA adduct standards have contributed to this uncertainty (*16*−*27*). Regardless of these discrepancies, a common feature of all applied assays is the detection of tamoxifen−DNA adducts in the overall genome but not at the level of nucleotide resolution. The latter is of concern because many genotoxic carcinogens leave a unique DNA adduction pattern and a signature mutation, which coincide at specific nucleotide sequence(s) in cancer-related genes (*14*, *15*). Consequently, mapping of DNA adducts in combination with mutation spectrometry in oncogenes and/or tumor suppressor genes can be used to investigate the etiology of cancers in which genotoxins are implicated (*14*, *15*).

The present study is the first comprehensive investigation of tamoxifen DNA adduct-targeted mutagenicity at the level of nucleotide resolution in a chromosomally integrated transgene, and a thorough mapping of tamoxifen-induced DNA adducts in an endogenous tumor suppressor gene of the human genome. Here, we investigated DNA adduction and induction of *cII* mutagenesis in Big Blue mouse embryonic fibroblasts treated *in vitro* with tamoxifen. In addition, we mapped the formation of DNA adducts in the *p53* gene in SV40 immortalized human hepatocytes and human endometrial carcinoma cells treated *in vitro* with tamoxifen. The *p53* tumor suppressor gene is one of the most frequently mutated genes of the human genome in a variety of cancers, including hepatic and endometrial cancers. The sequence-specific DNA-binding domain of this gene spanning exons 5−8 is the main target in various types of human cancers (*28*−*30*). We utilized terminal transferase-dependent polymerase chain reaction (TD-PCR)[1] for quantification of DNA adducts in both the *cII* transgene and *p53* gene and used a λ phage-based assay for determination of *cII* mutant frequency. We performed DNA-sequencing analysis to establish the spectrum of induced mutations in the *cII* transgene.

[1] Abbreviations: DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; *hprt*, hypoxanthine-guanine phosphoribosyl transferase; TD-PCR, terminal transferase-dependent polymerase chain reaction.

MATERIALS AND METHODS

Cell Culture and Chemical Treatment. Early passage embryonic fibroblasts were prepared from 13.5-day-old embryos of Big Blue mice (Stratagene, La Jolla, CA) as described earlier (*31*). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal bovine serum. SV40 immortalized human hepatocytes (THLE-3) and human endometrial carcinoma cells (RL95-2) were purchased from the American Type Culture Collection (Manassas, VA). The respective cells were grown in Small Airway Cell Basal Medium (Cambrex, Walkersville, MD) and Ham's F-12 (Irvine Scientific) supplemented with 10% fetal bovine serum. Once the cultures reached approximately 50% confluence, the media were removed and replaced with the respective serum-free media for 8 h prior to all treatments. The cultures were subsequently incubated with various concentrations of tamoxifen or control solvent, dimethyl sulfoxide (DMSO), for 24 h in the dark. To determine cell viability, representative cultures were trypsinized and harvested immediately after the 24-h treatment and evaluated by the trypan blue dye exclusion assay, thereafter. Other representative cultures were similarly processed at the end of the 24-h treatment and kept at −80 °C awaiting DNA isolation and adduct mapping analysis. A last set of representative cultures (mouse fibroblasts only) underwent multiple washes with phosphate-buffered saline after the 24-h treatment, fed with serum plus DMEM, and were passed twice during an 8-day growing period. The cultures were harvested as mentioned above and preserved at −80 °C awaiting DNA isolation and mutation analysis. The 8-day growing period is essential for the fixation of all induced mutations in the genome. All experimental treatments were run in the presence and absence of an external metabolic activating system according to standardized protocols (*32*, *33*). The system consisted of a reaction mixture containing 0.05 M HEPES/NaOH at pH 7.4, 0.5 mM NADPH, 5 mM $MgCl_2$, and 0.6 mg of rat liver S9 extract comprised of multiple cytochrome P450 and sulfotransferase enzymes (BD Gentest, Woburn, MA). All experimental conditions were in triplicate.

Genomic DNA Isolation. Genomic DNA was isolated using a standard phenol-based extraction and ethanol precipitation protocol (*34*). The DNA was dissolved in TE buffer (10 mM Tris-HCl and 1 mM EDTA at pH 7.5) and kept at −80 °C until further analysis.

TD-PCR for Mapping of DNA Adducts. The entire length of the *cII* transgene and exons 5−8 of the human *p53* gene were subjected to TD-PCR (for nontranscribed strands) as described earlier, with some modifications (*35*). Briefly, genomic DNA was used as a template, and single-stranded products were made by repeated primer extension. The extension protocol consisted of a custom-made biotinylated primer in a mixture of Vent$^{(exo-)}$ DNA polymerase (New England Biolabs, Beverly, MA). All information on primer design is available in Table 1. The thermocycler settings were as follows: 2 min at 95 °C, 2 min at 60 °C, 3 min at 72 °C, 10 cycles of (45 s at 95 °C, 2 min at 60 °C, and 3 min at 72 °C), 1 min at 95 °C, 2 min at 60 °C, and 10 min at 72 °C. The resulting product was mixed with streptavidin-coupled magnetic beads (Dynal Biotech ASA, Oslo, Norway), and

Table 1: Synthetic Oligomer Primers Used for TD-PCR Analysis of the *cII* Transgene and the Human *p53* Gene (Nontranscribed Stands)[a]

primer[b]		designation	t_m (°C)	bases	sequence
cII transgene		P1	59.8	27	5′-CAACAGCATAAATAACCCCGCTCTTAC-3′
		P2	63.2	24	5′-CCGCTCTTACACATTCCAGCCCTG-3′
		P3	64.9	26	5′-ACATTCCAGCCCTGAAAAAGGGCATC-3′
human *p53* gene	exon 5	5−4	56.6	16	5′-TGGGGACCCTGGGAA-3′
		5−5	61.3	20	5′-GCAACCAGCCCTGTCGTCTC-3′
		5−6	64.6	23	5′-TCTCTCCAGCCCCAGCTGCTCAC-3′
	exon 6	6−4	57.9	18	5′-AGGGCCACTGACAACCAC-3′
		6−5	61.0	24	5′-ACTGACAACCACCCTTAACCCCTC-3′
		6−6	63.0	22	5′-TAACCCCTCCTCCCAGAGACCC-3′
	exon 7	7−4	56.6	16	5′-GCAAGCAGAGGCTGGG-3′
		7−5	61.4	19	5′-TGGGGCACAGCAGGCCAGT-3′
		7−6	64.6	23	5′-CACAGCAGGCCAGTGTGCAGGGT-3′
	exon 8	8−4	55.9	29	5′-CAAGGAAAGGGTGATAAAAGTGAATCTGAG-3′
		8−5	62.9	28	5′-AAAGTGAATCTGAGGCATAACTGCACCC-3′
		8−6	66.1	24	5′-CTTGGTCTCCTCCACCGCTTCTTG-3′

[a] Each set consists of an oligonucleotide for primer extension (P1, 5−4, 6−4, 7−4, and 8−4; biotinylated primers), exponential PCR amplification (P2, 5−5, 6−5, 7−5, and 8−5; HPLC-purified primers), and labeling (P3, 5−6, 6−6, 7−6, and 8−6; fluorescence infrared dye-labeled primers) (IRD-700; LI-COR, Lincoln, NE). [b] All primers are designed using the OLIGO 4.0 program.

the mixture was gently rotated for 45 min at room temperature to allow primer extension products to bind. The magnetic bead-bound DNA was incubated with 0.15 M NaOH at 37 °C for 10 min. The beads were thoroughly washed with 1× TE at pH 7.5 in a magnetic particle concentrator (Dynal ASA), and the bead-bound single-stranded DNA was resuspended in 0.1× TE at pH 7.5 and subjected to homopolymeric ribotailing and adapter ligation (*36*). The ligation product was washed in the magnetic particle concentrator with 1× TE at pH 8.0, resuspended in 0.1× TE at pH 8.0, and PCR-amplified by using a second specific primer and a LP25 linker primer (*36*) in an Expand Long Polymerase mixture (Roche, Indianapolis, IN). The thermocycler was set for 2 min at 95 °C, 2 min at 61 °C, 3 min at 72 °C, 21 cycles of (45 s at 95 °C, 2 min at 61 °C, and 3 min at 72 °C), 45 s at 95 °C, 2 min at 61 °C, and 10 min at 72 °C. Labeling of the PCR products was achieved in an extension reaction mixture containing a fluorescence infrared dye-labeled primer (IRD-700; LI-COR, Lincoln, NE) and Expand Long Polymerase mix. The thermocycler settings were 2 min at 95 °C, 2 min at 65 °C, 3 min at 72 °C, 6 cycles of (45 s at 95 °C, 2 min at 65 °C, and 3 min at 72 °C), 1 min at 95 °C, 2 min at 60 °C, and 10 min at 72 °C. The labeled products were subjected to electrophoresis on a 5% polyacrylamide−urea gel in an IR^2 Long Ranger 4200 system (LI-COR) with simultaneous detection. Polymerase blocking DNA adducts were identified as specific bands along the sequencing gel whose intensities reflect the frequencies of the lesions. An untreated DNA sample was included in all runs to correct for nonspecific background bands, e.g., strand breaks and abasic sites resulting from spontaneous depurination of DNA that cause polymerase arrest (*36*).

cII Mutant Frequency Analysis. Mutant frequency of the *cII* transgene was quantified by the "λ select-*cII* mutation detection system" for Big Blue rodents (Stratagene). The assay system is based on lysis/lysogenesis of a λ phage upon infection of an indicator *Escherichia coli* (*37*). The λ phages can multiply either lytically or lysogenically in the host *E. coli* depending on their status of cII transcription (*38*). The cII protein is essential for activating the cI repressor and λ integrase, both of which are necessary for lysogenization (*38*). Thus, the *E. coli* indicators that bear phages with a mutated *cII* undergo lysis and form visible plaques on a special agar lawn (*37*). In the Big Blue system, the λ LIZ shuttle vector, however, harbors a *cI857* temperature sensitive (*ts*) mutation that makes the $cI_{(ts)}$ protein labile at temperatures higher than 32 °C (*39*). Hence, all λ LIZ phages regardless of their status of *cII* mutation multiply lytically in the host *E. coli* at incubating temperatures exceeding 32 °C (nonselective condition) (*37*).

Briefly, the λ LIZ shuttle vectors were rescued from the genomic DNA of mouse embryonic fibroblasts and packaged into viable phage particles using the "λ LIZ transpack packaging extract" according to the instructions of the manufacturer (Stratagene). The phages were preadsorbed to G1250 *E. coli*, and the bacterial culture was plated on TB1 agar plates. The plates were incubated for 48 h at 24 °C (selective condition) or overnight at 37 °C (nonselective condition). The *cII* mutant frequency was expressed as the ratio of the number of plaques formed under the selective condition to that formed under the nonselective condition. As recommended by the manufacturer, a minimum of 3×10^5 rescued phages was screened for each experimental condition. For quality assurance, control phage solutions containing mutant and wild-type λ *cII* with known mutant frequencies were assayed in all runs (Stratagene).

cII Mutation Spectrum Analysis. Putatively mutant *cII* plaques were all verified after being replated under the selective condition on a second TB1 agar plate. The verified plaques were subsequently amplified using the "λ select-*cII* sequencing primers" according to the recommended protocol of the manufacturer (Stratagene). The PCR products were purified with a QIA quick PCR-purification kit (QIAGEN GmbH, Hilden, Germany) and sequenced utilizing a Big Dye terminator cycle sequencing kit and an ABI-377 DNA Sequencer (ABI Prism, PE Applied BioSystems, Foster City, CA).

Statistical Analysis. Results are expressed as medians ± SE. All variables in treated versus control groups were compared by the Wilcoxon signed rank test. Comparisons among several groups were made by the Kruskall−Wallis one-way analysis of variance. The entire mutation spectra and the specific types of mutation in the treatment versus

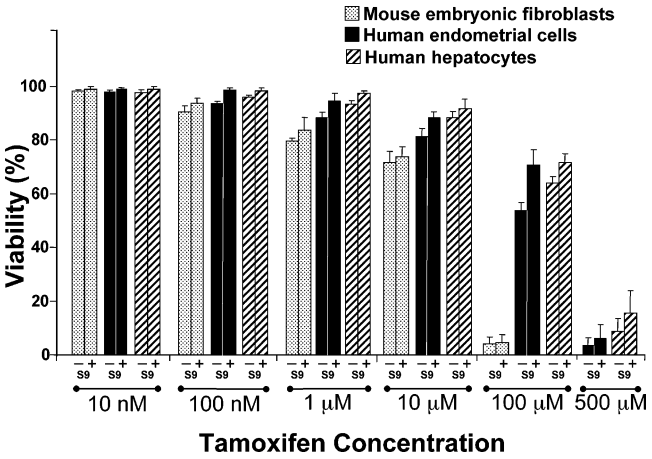

FIGURE 1: Cytotoxicity of tamoxifen in Big Blue mouse embryonic fibroblasts, immortalized human hepatocytes, and human endometrial carcinoma cells. Confluent cell cultures were treated with increasing concentrations of tamoxifen or control solvent, DMSO, for 24 h in the presence and absence of the S9 mix. Cell viability was determined by the trypan blue exclusion assay. Viability is expressed as a percentage of the total cell number. Results are expressed as medians of three independent experiments, with each experiment run in duplicate. Error bars = SE.

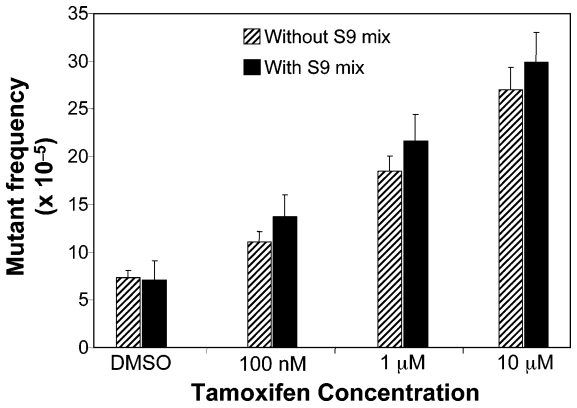

FIGURE 2: Mutant frequency of the *cII* transgene in Big Blue mouse embryonic fibroblasts treated with increasing concentrations of tamoxifen or control solvent, DMSO, for 24 h in the presence and absence of the S9 mix. Quantification was done 8 days after treatments using the "λ select-*cII* mutation detection system" for Big Blue rodents (Stratagene). This system permits detection of mutations within the *cII* transgene based on plaque formation. Mutant frequency was determined from a minimum of 3×10^5 plaques. Results are expressed as medians of three independent experiments, with each experiment run in duplicate. Error bars = SE.

control groups were compared by the hypergeometric test of Adams and Skopek (*40*) and the χ^2 test, respectively. All statistical tests were two-sided. Values of $p \leq 0.05$ were considered statistically significant.

RESULTS

Cytotoxicity Examination. We determined tamoxifen cytotoxicity in Big Blue mouse embryonic fibroblasts, immortalized human hepatocytes, and human endometrial carcinoma cells applying the trypan blue dye exclusion assay. The cells were treated with increasing concentrations of tamoxifen (10 nM, 100 nM, 1 μM, 10 μM, 100 μM, and 500 μM) for 24 h in the presence and absence of an external metabolic activating system (for simplicity, referred to as "the S9 mix"). Appreciable cytotoxic effects were observed in the micromolar dose range of tamoxifen in a dose-dependent fashion in all three cell types (Figure 1). Tamoxifen cytotoxicity was greatest in the mouse fibroblasts and least in the immortalized human hepatocytes, at all comparable doses. The severity of tamoxifen cytotoxicity was modestly lowered consequent to a S9 mix co-treatment in all of the three cell types at all tested doses (Figure 1).

cII Mutant Frequency and Mutation Spectrum Analyses. On the basis of the cytotoxicity of tamoxifen in Big Blue mouse embryonic fibroblasts, we determined its mutagenicity in the *cII* transgene at concentrations of 100 nM, 1 μM, and 10 μM, in the presence and absence of the S9 mix. As shown in Figure 2, tamoxifen was dose-dependently and significantly mutagenic even without a S9 mix co-treatment ($p < 0.01$). In the presence of the S9 mix, however, tamoxifen was slightly more mutagenic at all tested doses. Tamoxifen elevated the *cII* mutant frequency up to 3.7- and 4.1-fold over the background in the absence and presence of the S9 mix, respectively (Figure 2).

For mutation spectra analyses, DNA sequencing was performed on the *cII* mutant plaques obtained from cultures dosed with 10 μM tamoxifen (without a S9 mix co-treatment) or from control DMSO-treated cultures (number of sequenced plaques = 155 and 150, respectively). Overall, DNA sequencing confirmed a mutated *cII* in >97% of all analyzed plaques. Of these, the vast majority were single-base substitutions and less frequently insertions/deletions (Figure 3). In both induced and spontaneous mutation spectrum, there were two "jackpot" mutations at nucleotide positions 179−184 (G insertion/deletion) and 211 (G → C transversion) (Figure 3). These jackpot mutations are common phenomena in transgenic rodents and are consistently reported by us (*31*, *35*, *41*) as well as by others (*42*, *43*). Such mutations are thought to occur during early stages of development in the transgenic rodents, e.g., they are equivalent to hotspots of spontaneous mutagenesis. Consequent to clonal expansion, these jackpot mutations are widely found in various organs of untreated animals (*44*, *45*). To rule out the overrepresentation of jackpot mutations, it is methodologically appropriate to exclude them from all mutation spectra analyses. After the jackpot mutations were excluded, the spectrum of mutations induced by tamoxifen was significantly different from the control spontaneous mutation spectrum ($p < 0.0001$).

In Big Blue rodents, the *cII* transgene is almost certainly not transcribed after being integrated into the genome (*44*, *45*). Thus, it is unlikely that strand-biased mutagenesis, a phenomenon caused by transcription-coupled DNA repair in mammalian endogenous genes (*46*, *47*), occurs in this transgenic system. Therefore, we combined the strand mirror counterparts of all transitions (e.g., G → A + C → T) and transversions (i.e., G → T + C → A and G → C + C → G) and compared the specific types of mutations between induced and spontaneous mutation spectra. G:C → T:A transversions were the hallmark mutations induced by tamoxifen as compared to the control (23.2 versus 12.5%, $p < 0.03$) (Figure 4). On the basis of the fact that the *cII* transgene of the Big Blue mouse system is almost fully methylated at all CpG dinucleotides (*48*), the induced G:C → T:A did not, however, occur preferentially at methylated CpG dinucleotides (3.6 versus 4.2% in the control, $p < 0.8$) nor did the overall induced transitions and transversions favor

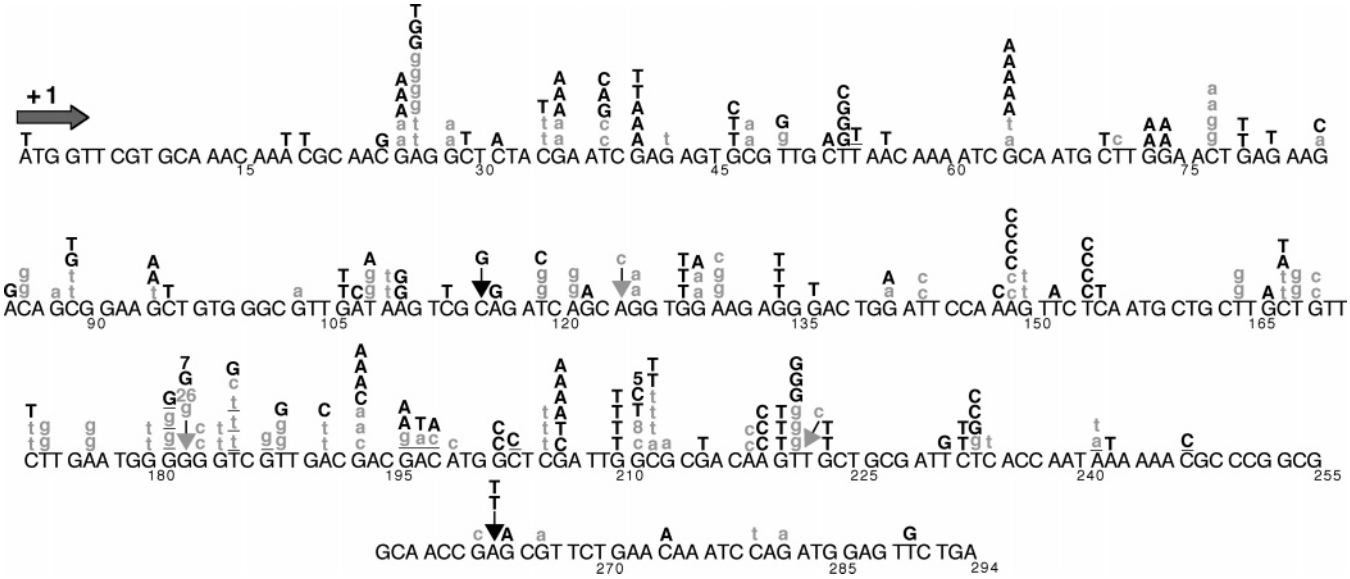

FIGURE 3: Detailed mutation spectra of the *cII* transgene in Big Blue mouse embryonic fibroblasts treated with 10 μM tamoxifen or control solvent, DMSO, for 24 h in the absence of the S9 mix (total number of sequenced mutant plaques = 155 and 150, respectively). Tamoxifen-induced mutations and spontaneous mutations are in black (capital letters) and gray (small letters) typefaces, respectively. Substituted bases are in bold. Deleted bases are underlined; multiple base deletions are continuously underlined. Inserted bases are shown with an arrow. Mutation multiplicity is shown by the number above the base, e.g., $\underline{C}^8 = 8$ occurrences of cytosine deletion. Numbers below the bases are the nucleotide positions.

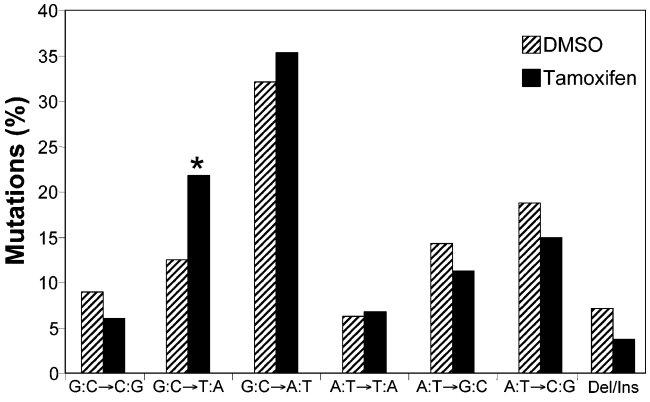

FIGURE 4: Comparative mutation spectra of the *cII* transgene in Big Blue mouse embryonic fibroblasts treated with 10 μM tamoxifen or control solvent, DMSO, for 24 h in the absence of the S9 mix (total number of sequenced mutant plaques = 155 and 150, respectively). For comparison, the strand mirror counterparts of all transitions (e.g., G → A + C → T) and transversions (e.g., G → T + C → A and G → C + C → G) were combined in each group. Ins = insertion; Del = deletion. (*) As compared with "nontreated control"; $p < 0.03$.

a significant bias at methylated CpGs (33.9 versus 23.9% in the control, $p < 0.1$).

DNA Adducts Mapping. On the basis of our data on tamoxifen cytotoxicity and mutagenicity, we selected 10−100 μM doses of tamoxifen as the biologically relevant doses for DNA-adduct mapping experiments. Tamoxifen in this dose range gives rise to almost equivalent amounts of α-hydroxy-tamoxifen metabolites (*2*, *17*) as those measured in the blood serum of tamoxifen-treated patients (*49*). As shown in Figure 5, tamoxifen treatment of Big Blue mouse embryonic fibroblasts at a concentration of 10 μM yielded polymerase-blocking DNA adducts at multiple nucleotide positions along the *cII* transgene. Major tamoxifen−DNA adduction sites in the *cII* transgene were at nucleotide positions 1−15, 23−40, 44−54, 68−70, 76−77, 87−93, 100−110, 125−133, 140−142, 164−170, 178−183, 190−193, 218−220, 225−227, and 238−241 (Figure 5). A co-treatment with the S9 mix in these cells slightly intensified the extent of DNA adduction (Figure 5). Increasing the concentrations of the administered tamoxifen, e.g., up to 1 order of magnitude, did not considerably increase the formation of DNA adducts (with or without a S9 mix co-treatment, data not shown).

Immortalized human hepatocytes but not human endometrial carcinoma cells treated with tamoxifen (10 μM) showed a preferential formation of DNA adducts at specific codons in exon 6 and exon 8 of the *p53* gene (Figure 6). In the immortalized hepatocytes, DNA adduction was saturated in exon 6 even without a S9 mix co-treatment (Figure 6). However, the formation of DNA adducts in exon 8 was somewhat enhanced consequent to a S9 mix co-treatment (Figure 6). For the most part, a co-treatment of the endometrial carcinoma cells with the S9 mix did not appreciably induce DNA adduction (Figure 6). Raising the concentrations of the administered tamoxifen from 10 to 500 μM only marginally affected the formation of DNA adducts in the immortalized human hepatocytes and negligibly influenced DNA adduction in the human endometrial carcinoma cells (regardless of a co-treatment with the S9 mix, data not shown). In sequence context, the majority of DNA adduction sites in the *p53* gene were guanine-rich. On the basis of our previous observation of a complete and tissue-independent methylation of CpG dinucleotides along exons 5−8 of the human *p53* gene (*50*), the overall induced-DNA adducts in the *p53* gene were not preferentially formed at methylated CpG dinucleotides. As described in the caption for Figure 6, of all codons wherein DNA adduction occurred, only two in exon 6 and one in exon 8 contain CpGs. This is in good agreement with the observed tamoxifen-induced mutagenesis in the *cII* transgene, which was not biased at methylated CpGs either (Figure 3).

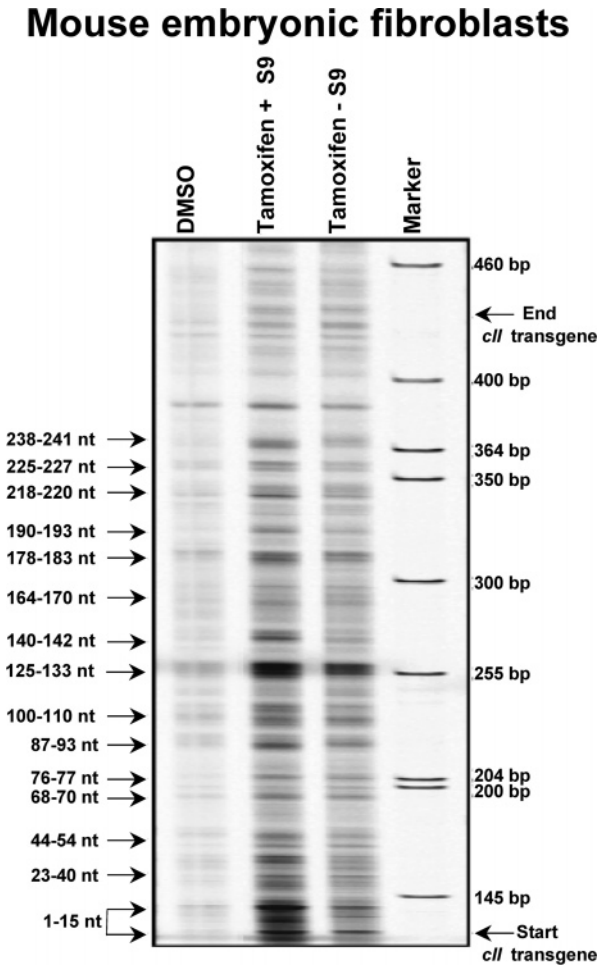

FIGURE 5: Mapping of DNA adducts in the *cII* transgene in Big Blue mouse embryonic fibroblasts treated with 10 μM tamoxifen or control solvent, DMSO, for 24 h in the presence and absence of the S9 mix. Genomic DNA was isolated and subjected to TD-PCR (for nontranscribed strand) (*35*). bp = base pair; nt = nucleotide position.

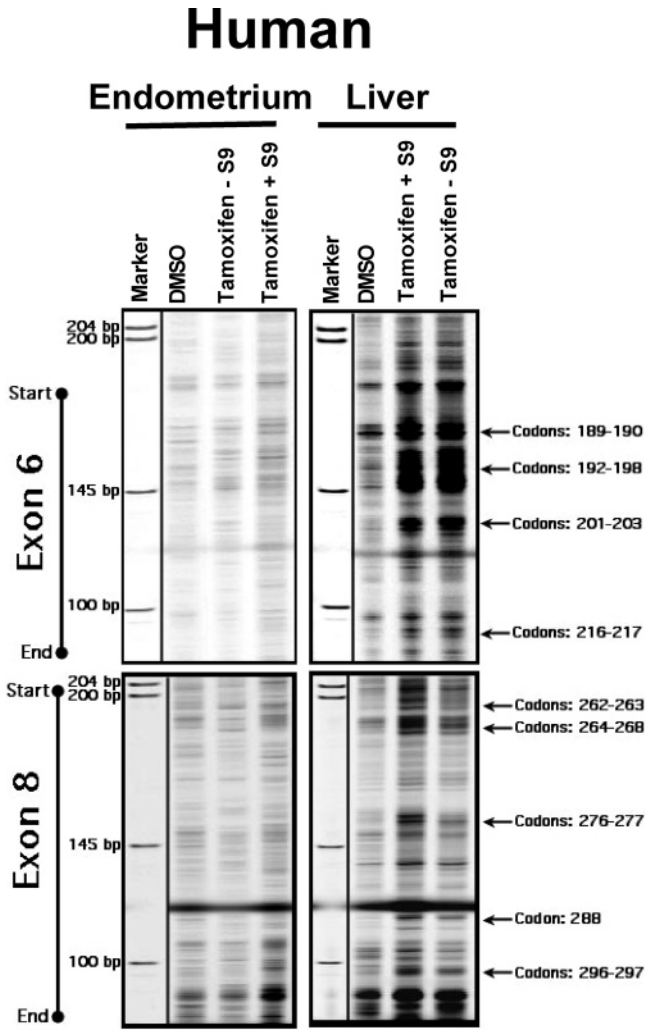

FIGURE 6: Mapping of DNA adducts in the *p53* gene in immortalized human hepatocytes and human endometrial carcinoma cells treated with 10 μM tamoxifen or control solvent, DMSO, for 24 h in the presence and absence of the S9 mix. Genomic DNA was isolated and subjected to TD-PCR (for nontranscribed strands) (*35*). Codon positions are indicated. bp = base pair; M = sizing standard. Of all codons wherein DNA adduction occurred, only two in exon 6 and one in exon 8 contain CpGs. However, the majority of DNA adduction sites in the *p53* gene were guanine-rich (see below). Sequence context of DNA adduction sites is as follows: exon 6, codons 189−190, 5′-**G**CCCCT-′3; codons 192−198, 5′-CA**G**-CATCTTATCC**GAGTGG**AA-3′; codons 201−203, 5′-TT**G**C**G**T-**G**T**G**-3′; codons 216−217, 5′-**G**T**GG**T**G**-3′; exon 8, codons 262−263, 5′-**GG**TAAT-3′; codons 264−268, 5′-CTACT**GGG**AC**GG**-3′; codons 276−277, 5′-**G**CCT**G**T-3′; codon 288, 5′-AAT-3′; codons 296−297, 5′-CACCAC-3′.

DISCUSSION

Tamoxifen is an extensively used drug for chemotherapy and chemoprevention of breast cancer worldwide. Women's exposure to tamoxifen is, however, associated with an increased incidence of endometrial cancer. The cancer-causing effect of tamoxifen is equivocally ascribed to a genotoxic mechanism giving rise to promutagenic tamoxifen−DNA adducts (*1*−*3*). Here, we investigated DNA adduct-targeted mutagenicity of tamoxifen at the level of nucleotide resolution in the *cII* transgene in Big Blue mouse embryonic fibroblasts and mapped the formation of tamoxifen-induced DNA adducts in the *p53* tumor suppressor gene in SV40 immortalized human hepatocytes and human endometrial carcinoma cells.

Tamoxifen had a detrimental effect on viability of all three cell types mainly in the administered micromolar dose range. The cytotoxic effects of tamoxifen were dose-dependent throughout. A concurrent treatment with the S9 mix modestly reduced the cytotoxicity of tamoxifen in all cases (Figure 1). Presumably, adding of an external activating system to the metabolic machinery of the cell enhances the generation of reactants that are less cytotoxic than the parent compound, tamoxifen. Overall, mouse cells were more sensitive to tamoxifen cytotoxicity compared to human cells (Figure 1). This may reflect an interspecies variation in tamoxifen activation and/or detoxification.

Tamoxifen treatment of mouse embryonic fibroblasts significantly increased the *cII* mutant frequency, leaving a unique mutation spectrum and a signature mutation of G:C → T:A transversions on this transgene (Figures 2−4). The induction of *cII* mutant frequency was dose-dependent and slightly augmented consequent to a co-treatment with the S9 mix at all tested doses (Figure 2). This implies a relatively efficient metabolic capacity of these cells for converting tamoxifen to promutagenic species. Similar characteristic mutagenicity has previously been reported for tamoxifen both *in vitro* and *in vivo*. Analogous mutagenic potency and specificity of tamoxifen has been shown in the *lacI* and *cII*

transgenes in rats (*7*, *8*, *43*, *51*−*53*), as well as in the hypoxanthine-guanine phosphoribosyl transferase (*hprt*) gene in Chinese hamster cells (*54*). A single study has, however, reported a distinct *p53* mutation spectrum in tamoxifen-induced rat liver tumors (*55*). Of all analyzed tumors in this study, half (12 of 24) harbored *p53* mutations in the highly conserved region encompassing exons 5−9. Nine contained a miscoding A → G transition at codon 231. The remainder had a nonmiscoding C → T transition at codon 294 or simultaneous A → G and C → T mutations at codons 231 and 294, respectively. Critics have, however, dismissed these results, suspecting an erroneous pseudogene amplification by the applied methodology in this study (*1*, *2*). The findings have alternatively been ascribed to a polymorphism specific to the particular strain of rats used in that study (normal tissues from the affected animals were not analyzed) (*1*, *2*).

A major DNA adduct induced by tamoxifen is identified as α-(N^2-deoxyguanosinyl)-tamoxifen both *in vitro* and *in vivo* (*56*−*60*). This stable DNA adduct is poorly to moderately reparable and possesses a great miscoding potential. Molecular-modeling studies have predicted a unique mutagenic potential for this DNA adduct, i.e., G → T and G → C transversions and deletions (*61*−*63*). Site-specific mutagenicity of trans and cis diastereoisomers of this DNA adduct has shown G → T transversions as the predominant type of induced mutations (*23*, *64*). Additional G → A transitions and G → C transversions have also been reported (*52*). A characteristic G → T transversion has prevailed in the spectra of tamoxifen-induced mutations in the *lacI* and *cII* transgenes (*7*, *8*, *43*, *51*−*53*) as well as in the endogenous *hprt* gene (*54*). Furthermore, tamoxifen and its derivatives have been shown to cause oxidative stress generating 8-hydroxydeoxyguanosine (*65*). This oxidized base adduct has a signature mutation of G → T transversions in a variety of test systems (*41*, *66*, *67*). In our hands, the potency of tamoxifen mutagenicity was in good agreement with the extent of its DNA adduction in the *cII* transgene. Accordingly, tamoxifen exhibited higher mutagenicity in the presence of the S9 mix relative to its absence at all comparable doses (Figure 2). At the same time, tamoxifen-induced DNA adduction was intensified consequent to a co-treatment with the S9 mix (Figure 5). Moreover, the specificity of tamoxifen mutagenicity followed the mutagenic potential of its induced-DNA adducts. Tamoxifen produced a unique mutation spectrum in the *cII* transgene with an excess of G:C → T:A transversions (Figure 4); however, there was no clear-cut mutation hotspot (Figure 3). The latter might be explained by the multitude of DNA adducts formed almost evenly throughout this transgene (Figure 5).

Tamoxifen-induced DNA adducts were formed preferentially at specific codons along exons 6 and 8 of the *p53* tumor suppressor gene in immortalized human hepatocytes but not human endometrial carcinoma cells (Figure 6). A co-treatment with the S9 mix enhanced DNA adduction in exon 8 in the immortalized hepatocytes; however, it was only negligibly effective in the endometrial carcinoma cells. Hepatic DNA adduction was saturated in exon 6 and readily detectable in exon 8 even without the aid of an external activating system. On one hand, these findings suggest that human liver is proficient for metabolic activation of tamoxifen and generation of DNA adduct-inducing reactants. On the other hand, they imply an inefficient activation and/or efficient detoxification of tamoxifen in human endometrium. The liver is the major site of tamoxifen biotransformation in humans. This organ contains a variety of enzymes required for activation of tamoxifen, e.g., various isoforms of cytochrome p450 and sulfotransferase 2 (*1*, *2*). Human endometrium expresses substantial levels of UDP-glucuronosyltransferases, especially UGT1As (*68*, *69*) that catalyze conjugation of tamoxifen derivatives to glucuronic acid, thereby yielding excreteable end products (*70*). In human endometrium, the detoxification pathway of tamoxifen seems to overwhelm its activation pathway. This idea gains support from our observation that even the adding of an external activating system did not appreciably influence DNA adduction in the human endometrial carcinoma cells (Figure 6). Our overall findings confirm the previously determined tamoxifen−DNA adducts in the genomic DNA of the liver (*24*) and the nondetectability or equivocal detectability of these adducts in the genomic DNA of the endometrium (*16*, *17*, *25*−*27*) from tamoxifen-treated patients.

The lack of genotoxicity of tamoxifen in human endometrial carcinoma cells is suggestive of an underlying estrogenic (agonist) mechanism for tamoxifen-associated endometrial cancer in humans. However, the verified genotoxicity of tamoxifen in immortalized human hepatocytes in view of the noncarcinogenicity of this drug in human liver underscores the interdependence of multiple stages involved in carcinogenesis. Although DNA adduction is a triggering factor in the initiation stage of carcinogenesis, other influential factors, e.g., cellular proliferation, are required to advance this multistage process, i.e., by translating DNA adducts to genetic alterations such as mutations in cancer-related genes (*13*). Mitotically, the liver becomes an essentially quiescent organ in adulthood. Hence, tamoxifen−DNA adduction in the liver may paradoxically prevent, at least partially, its reactants from reaching highly dividing organs via, e.g., circulating blood. It is conceivable that tamoxifen−DNA adducts per se in the liver may not have as significant biological consequences as they might have in a greatly proliferative organ. Notwithstanding is that a great number of carcinogens is metabolized in the liver to DNA adduct-inducing reactants, yet not many of them cause liver tumorigenesis.

Last, we acknowledge that our *in vitro* findings in immortalized human hepatocytes and endometrial carcinoma cell lines may not exactly mirror the *in vivo* situation in human liver and endometrium. Specifically, we recognize that the metabolic activation and detoxification of tamoxifen in these cell lines might not necessarily be similar to those in normal human hepatocytes and endometrial cells. However, we should also state that both of these two cell lines have been thoroughly characterized and extensively validated as invaluable models to study xenobiotic metabolism and toxicity. The hepatocyte cell line used here is a SV40 large T antigen-immortalized human liver cell line (*71*). This cell line has proven to metabolize a variety of compounds including benzo[*a*]pyrene, *N*-nitrosodimethylamine, and aflatoxin B1 to their ultimate carcinogenic forms that adduct DNA (*71*). This cell line possesses functional cytochrome P450 isoenzymes, and retains various other enzymes involved in biotransformation of chemical carcinogens, e.g., epoxide hydrolase, NADPH cytochrome P450 reductase, superoxide dismutase, catalase, glutathione *S*-transferases,

and glutathione peroxidase (*71*, *72*). The endometrial cell line used here is an exhaustively defined carcinoma cell line (estrogen receptor positive) (*73*). This cell line has too been verified as a relevant model for studying human endometrial neoplasia (*74*, *75*). The cell line has an established capacity for metabolizing estradiol and estrone through sulfoconjugation (*76*, *77*). This cell line contains multiple cytochrome p450 isoenzymes, it is highly responsive to benzo[*a*]pyrene and halogenated aromatic hydrocarbons in terms of induction of cytochrome p450 1A/1B (*78*, *79*). Altogether, the wealth of information available on these two cell lines justifies their use as appropriate *in vitro* model systems for investigating the etiology of human cancers.

One might argue that, in principle, primary cell cultures are better representatives than transformed cell lines for unraveling the *in vivo* metabolic activation and/or detoxification of xenobiotics. However, it is known that tamoxifen is activated and detoxified by multiple enzymes many of which are polymorphic in humans (*80*−*84*). Therefore, a small number of primary cell cultures prepared from randomly selected human subjects cannot necessarily represent the *in vivo* situation for the general population. For example, Phillips et al. (*85*) were unable to detect tamoxifen-induced DNA adducts in the primary cultures of human hepatocytes using the ^{32}P-postlabeling assay. On the other hand, Pathak and Bodell (*86*) have demonstrated that human liver microsomal preparations can activate tamoxifen and, subsequently, cause DNA adduction in calf thymus DNA using a similar methodology. In the present study, we have ensured the metabolic conversion of tamoxifen to DNA reactive derivatives in all three cell types by applying a standardized external metabolic activating system, which is proven to generate tamoxifen metabolites capable of forming DNA adducts (*32*, *33*). Ideally, conducting a large-scale population-based study in which primary cell cultures are used to investigate the effects of tamoxifen will be an informative approach to study tamoxifen carcinogenicity. However, such investigation is far beyond the scope of a pilot study such as the present one.

In summary, we have shown a characteristic DNA adduct-targeted mutagenicity of tamoxifen in the *cII* transgene in Big Blue mouse embryonic fibroblasts. We have also verified tamoxifen genotoxicity based on its DNA adduction property in the *p53* tumor suppressor gene in immortalized human hepatocytes but not human endometrial carcinoma cells. We conclude that a nongenotoxic mechanism, possibly through the estrogen agonist effect, might be involved in tamoxifen-attributable human endometrial cancer.

ACKNOWLEDGMENT

We thank Prof. Dr. Arthur Riggs and Dr. Hsiu-Hua Chen for sharing TD-PCR expertise and Steven Bates for assisting in cell culturing.

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BI0503753