DNA adduct formation and mutant induction in Sprague–Dawley rats treated with tamoxifen and its derivatives

Gonçalo Gamboa da Costa, L.Patrice McDaniel-Hamilton, Robert H.Heflich, M.Matilde Marques and Frederick A.Beland

The non-steroidal anti-estrogen tamoxifen is used as an adjunct chemotherapeutic agent for the treatment of all stages of breast cancer and more recently as a chemoprotective agent in women with elevated risk of developing breast cancer. While beneficial for the treatment of breast cancer, tamoxifen increases the risk of endometrial cancer. In addition, it has been shown to induce liver and endometrial tumors in rats. Tamoxifen is genotoxic in rat liver, as indicated by the formation of DNA adducts, through a metabolic pathway involving the α-hydroxylation of tamoxifen and N-desmethyltamoxifen. Since the contribution of these α-hydroxy metabolites of tamoxifen to the induction of endometrial tumors is presently unknown, we compared the extent of DNA adduct formation in liver and selected non-hepatic tissues of female Sprague–Dawley rats treated by gavage with tamoxifen, α-hydroxytamoxifen, N-desmethytamoxifen, α-hydroxy-N-desmethyltamoxifen and N,N-didesmethytamoxifen, or intraperitoneal injection with tamoxifen, α-hydroxytamoxifen, 3-hydroxytamoxifen and 4-hydroxytamoxifen. In addition, spleen lymphocytes from rats treated by gavage with tamoxifen or α-hydroxytamoxifen were assayed for the induction of mutants in the hypoxanthine phosphoribosyl transferase (Hprt) gene. The relative levels of binding in rats treated by gavage were α-hydroxytamoxifen ≈ tamoxifen ≈ N-desmethyltamoxifen ≈ α-hydroxy-N-desmethyltamoxifen > N,N-didesmethytamoxifen. In rats dosed intraperitoneally, the relative order of binding was α-hydroxytamoxifen > tamoxifen > α-hydroxy-N-desmethyltamoxifen > 3-hydroxytamoxifen. None of the compounds resulted in an increase in DNA adducts in uterus, spleen, thymus or bone marrow DNA from rats treated by gavage or in uterus DNA from rats injected intraperitoneally. Neither tamoxifen nor α-hydroxytamoxifen increased the Hprt mutant frequency in spleen T-lymphocytes. These results confirm previous observations that tamoxifen is activated to a genotoxic agent in rat liver through α-hydroxylation, and also suggest that endometrial tumors in rats do not arise from the formation of tamoxifen–DNA adducts.

Introduction

Tamoxifen is an important adjunct chemotherapeutic agent for treating women with breast cancer. In clinical trials involving >30 000 women, it decreased the reoccurrence of breast cancer by up to 50% (1). More recently, this anti-estrogen has been given to women at increased risk of developing breast cancer, with a resultant 50% decrease in the occurrence of invasive breast cancer (2). A potential complication from the use of tamoxifen is the induction of endometrial cancer. When used as a chemotherapeutic agent, it produced up to a 4-fold increase in endometrial tumors (1); when used prophylactically, the risk increased 2.5-fold (2). The mechanism for the induction of these tumors is not known.

In addition to causing endometrial cancer in women, tamoxifen is carcinogenic in rodents. In rats treated orally, tamoxifen induced a high incidence of hepatic tumors (3–5), and endometrial tumors have been observed in rats and mice following transplacental, neonatal and/or chronic exposure (6–10). There is substantial evidence that the hepatic tumors in rats are due to a genotoxic mechanism resulting from the formation of tamoxifen–DNA adducts (4,11–15); however, as with humans, the mechanism for the induction of endometrial tumors in rodents has not been established, although hormonal imprinting (10) and genotoxic (6,7) mechanisms have been proposed.

In rat liver, tamoxifen is activated to an electrophile by sequential α-hydroxylation and esterification (16–22; Figure 1). The α-hydroxylation is catalyzed primarily by cytochrome P450 3A4 (23), while esterification appears to occur mainly through sulfation, which is catalyzed by sulfotransferases, in particular SULT2A (20–22,24). The major DNA adduct resulting from this metabolism is (E)-α-(deoxyguanosin-N²-yl)tamoxifen, which is accompanied by minor amounts of the Z diastereomer and deoxyadenosine adducts (25–27). Another major activation pathway for tamoxifen in rat liver is N-desmethylation followed by α-hydroxylation (or α-hydroxylation followed by N-desmethylation) to give α-hydroxy-N-desmethyltamoxifen, which is presumably then esterified (28–31; Figure 1). The major adduct resulting from this pathway has recently been characterized as (E)-α-(deoxyguanosin-N²-yl)-N-desmethyltamoxifen (32). Both this adduct and its Z diastereomer have subsequently been identified in the reaction of α-acetoxy-N-desmethyltamoxifen with deoxyguanosine (33).

An additional metabolic pathway proposed for the activation of tamoxifen is sequential oxidation to 4-hydroxytamoxifen.
Fig. 1. Metabolic activation pathways for tamoxifen. Only the major adducts resulting from α-hydroxytamoxifen, 4-hydroxytamoxifen and α-hydroxy-N-desmethyltamoxifen are shown. DNA adducts from 4-hydroxytamoxifen have not been detected in vivo.

and 4-hydroxytamoxifen quinone methide (34–37; Figure 1). Reaction of 4-hydroxytamoxifen quinone methide with DNA in vitro leads to the formation of (E) and (Z)-α-(deoxyguanosin-N2-yl)-4-hydroxytamoxifen (38); nonetheless, these adducts were not detected in hepatic DNA upon administration of 4-hydroxytamoxifen to rats (39,40). In addition, the cutaneous application of 4-hydroxytamoxifen to rats did not induce tumors (41).

Although activation pathways of tamoxifen in rat liver have been established, the contribution of the α-hydroxy metabolites of tamoxifen to the toxicities observed in non-hepatic tissues (e.g. endometrial tumors) is presently unknown. Accordingly, we have compared the extent of DNA adduct formation in the livers and selected non-hepatic tissues of female Sprague–Dawley rats treated by gavage with tamoxifen, α-hydroxytamoxifen, N-desmethyaltamoxifen, α-hydroxy-N-desmethyltamoxifen and N,N-didesmethyltamoxifen. In addition, spleen lymphocytes from rats treated with tamoxifen or α-hydroxytamoxifen were assayed for the induction of mutants in the hypoxanthine phosphoribosyl transferase (Hprt) gene.

The route of drug administration may have an important bearing on the metabolic activation pathways of tamoxifen. For example, oral dosing with tamoxifen resulted in decreased uterine peroxidase activity (39), whereas intraperitoneal administration caused a 10-fold induction of peroxidase activity (37). Although uterine DNA adducts were not detected after oral dosing with tamoxifen (15,39), the induction of peroxidase activity by intraperitoneal treatment could result in an increased production of 4-hydroxytamoxifen and its quinone methide. To test this possibility, a second experiment was conducted in which hepatic and uterine DNA adducts were assessed in tumors (41).

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Materials and methods

Chemicals

Tamoxifen, salmon testes DNA, bis(2-hydroxyethyl)iminotris(hydroxymethyl) methane (Bis-Tris), trioctanoin and the enzymes used in DNA hydrolysis were purchased from Sigma (St Louis, MO). Carrier-free [γ-32P]ATP
(7000 Cl/mmol) was produced from ICN Pharmaceuticals (Costa Mesa, CA). T4 polynucleotide kinase (PNK) was acquired from Amersham US Biochemical (Cleveland, OH). 3-Hydroxytamoxifen citrate was obtained from Klingen Pharma GmbH (Munich, Germany). All other commercially available reagents were purchased from Aldrich (Milwaukee, WI) or Sigma–Aldrich Química, S.A. (Madrid, Spain) and were used as received. Whenever necessary, solvents were purified by standard procedures (45).

**Instrumentation**

Melting points were measured with a Leica Galen III hot-stage apparatus and are uncorrected.

HPLC analyses were conducted with a μBondapak C18 column (3.9 mm × 30 cm; Waters, Milford, MA), using either a Varian system consisting of a Star 9012 ternary gradient pump and a Polychrom 9006S diode array spectrophotometric detector (Varian, Palo Alto, CA), equipped with a Rheodyne Model 7125 injector (Rheodyne, Cotati, CA), or a Waters system consisting of two Model 510 pumps and a Model 660 automated gradient controller, equipped with a Rheodyne Model 7125 injector and a Hewlett-Packard 1050 diode array spectrophotometric detector (Hewlett-Packard, Wilmington, DE).

UV absorbance was monitored at 254 or 280 nm.

HPLC analyses of [3H]-post-labeled samples were conducted with a 5 μm Delta-Pak C18 column (30.9 mm × 3.0 cm; Waters) using a Waters system, as described above, equipped with a Radiomatic Flo-One Model A-500 on-line radioactivity detector (Packard Instruments, Meriden, CT).

(E)-α-(Deoxyguanosin-N2)-tamoxifen was weighed to an accuracy of 0.1 μg with a Sartorius 4504 Mpb1 ultra-micro balance. An additional period of 30 min and then cooled to 0°C.

1H HPLC analyses of 32P-post-labeled samples were conducted with a 5 μm Hamilton PRP-X110 column (250 mm × 4.6 mm i.d.) in a Waters system consisting of an Aerotech solvent delivery system, a Waters 474 column oven, a Waters 600 pump, a Waters 331 solvent manager, a Waters 248 UV detector, a Waters 441 fluorescence detector, and a Waters 440 data module. A solution of 1-chloroethyl chloroformate (730 mg, 5.8 mmol) in 1,2-dichloroethane (7.5 ml) was added dropwise over a period of 10 min and the mixture was refluxed overnight. Following evaporation of the solvent, the residue was dissolved in methanol (20 ml) and refluxed for 45 min. The solvent then was evaporated, and the crude mixture was washed three times with n-hexane and dried under vacuum. N-Desmethyltamoxifen was recovered as the corresponding hydrochloride salt (516 mg, 97%).

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An excess of potassium t-butoxide (2.6 g, 22 mmol) in n-hexane (13 ml) kept under nitrogen at room temperature, were added sequentially n-propylbenzene (3 ml, 21.5 mmol), n-butyllithium (1.6 M in hexane, 19.2 mmol) and N,N′-tetramethylethylenediamine (6.5 ml, 43 mmol). The ensuing red suspension was stirred at room temperature for an additional period of 30 min and then cooled to ~70°C. A solution of 4-hydroxybenzophenone (2.5 g, 12.6 mmol) in tetrahydrofuran (20 ml) was subsequently added over ~45 min, and the mixture was allowed to reach room temperature. After 2 h, the reaction was quenched by addition of a saturated ammonium chloride solution (350 ml) and the organic materials were extracted with methylene chloride. The crude mixture of diastereomeric carbinals was dehydrated by treatment with a 1:1 solution of methanol and 25% H3SO4. Following flash chromatography on silica gel (Type 60; E. Merck, Darmstadt, Germany), using methylene chloride as the eluent, 1-(4-hydroxyphenyl)-1,2-diphenyl-1-one (2.28 g, 60%) was isolated as a 1:1 mixture of the E and Z isomers. 1H NMR (acetonitrile-d3): δ 0.89 (3H, t, J 7.5, CH3CH2), 3.05 (2H, t, J 7.5, CH2CH2), 3.97 (3H, s, OCH3), 7.13–7.15 (8H, m, Ph). MS (FAB): m/z 314 (M+H+), 316 (M+2H+), 326 (M+3H+), 380 (M+4H+), 392 (M+5H+).

(E,Z)-1-(4-Hydroxyphenyl)-1,2-diphenylbut-1-ene was prepared from 4-hydroxybenzophenone using super-base metalled propylene (48).

(E,Z)-1-(4-Hydroxyphenyl)-1,2-diphenylbut-1-ene was analyzed by HPLC using a 5 μm Bondapak C18 column (30.9 mm × 3.0 cm; Waters) using a Waters system, as described above, equipped with a Radiomatic Flo-One Model A-500 on-line radioactivity detector (Packard Instruments, Meriden, CT).

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DNA adduct standards

α-Acetoxytamoxifen was prepared from α-hydroxytamoxifen and reacted with DNA using the method of Osborne et al. (25), as modified by Beland et al. (39). Following sequential extraction of unbound materials with diethyl ether and n-butanol, both of which had been pumped at 5 mM Bis-Tris, 0.1 mM EDTA (pH 7.1), the modified DNA was precipitated with NaCl and ethanol, and redissolved in 5 mM Bis-Tris, 0.1 mM EDTA (pH 7.1) at a concentration of ~1 mg/ml. The DNA was hydrolyzed to nucleosides by treatment with DNase I, followed by alkaline phosphatase and phosphodiesterase I (49). The adducts were then partitioned into n-butanol, which had been pumped at 5 mM Bis-Tris, 0.1 mM EDTA (pH 7.1) and the n-butanol was evaporated. The residue was redissolved in methanol and purified by HPLC. The flow of 0.2 ml/min, using a 17 min linear gradient of 0–60% acetonitrile in 100 mM ammonium acetate (pH 5.7), followed by a 3 min linear gradient to 100% acetonitrile and a 5 min isocratic elution with acetonitrile. The major adduct, which has been characterized as (E)-α-(deoxyguanosin-Ν²-yl)tamoxifen (25), was collected and the sample was thoroughly evaporated under vacuum and weighed in a microbalance. Additional confirmation of this quantification was obtained by 1H NMR in methanol-d₄, using nitromethane as an internal standard. Based upon the UV absorbance of a 1.83×10⁻⁵ M methanolic solution, the molar extinction coefficients of the adduct were determined to be 16 800 and 13 200 M⁻¹cm⁻¹ at 250 and 275 nm, respectively.

To obtain DNA samples with different extents of modification, a series of reactions was conducted in which the amount of α-acetoxytamoxifen was varied from 1 mg/mg DNA to 0.01 μg/mg DNA. After purification, aliquots of the DNA samples from modifications conducted with 1, 0.1 and 0.01 mg α-acetoxytamoxifen/mg DNA were enzymatically hydrolyzed, as described above, and analyzed directly by HPLC without prior n-butanol extraction of the adducts. Based upon the molar extinction coefficient for (E)-α-(deoxyguanosin-Ν²-yl)tamoxifen, the extent of modification was 207, 58 and 3.9 adducts/10⁶ nucleotides, respectively. Modifications conducted at 1, 0.1 and 0.01 μg α-acetoxytamoxifen/mg DNA were hydrolyzed in a similar manner and analyzed by mass spectrometry (50), which indicated binding levels of 48, 5.2 and 0.59 adducts/10⁶ nucleotides, respectively.

α-Sulfoxide-N-desmethyltamoxifen was prepared from α-hydroxy-N-desmethyltamoxifen and reacted with DNA as detailed in Gamboa da Costa et al. (32). Following extraction of unbound materials and precipitation, the modified DNA was hydrolyzed to nucleosides as described above. HPLC analysis indicated one major adduct, which has been characterized as (E)-α-(deoxyguanosin-Ν²-yl)-N-desmethyltamoxifen (32).

4-Hydroxytamoxifen quinone methide was prepared from (E,Z)-4-hydroxytamoxifen and reacted with DNA as described in Marques and Beland (38). Two major adducts, which have been identified as (E)- and (Z)-α-(deoxyguanosin-Ν²-yl)-4-hydroxytamoxifen (38), were detected by HPLC.

Treatment of animals

Rats were treated according to the protocol of White et al. (12). Specifically, 12 female Sprague–Dawley rats (Crl:COBS CD (SD) BR outbred, 8 weeks old, 199 ± 20 g, obtained from the breeding colony at the National Center for Toxicological Research] were treated by gavage with seven daily doses of tamoxifen (20 mg/kg, 54 μmol/kg, dissolved in 200 μl trioctanoin). Additional animals were treated in the same manner with equimolar doses of α-hydroxytamoxifen, α-hydroxy-N-desmethyltamoxifen, N,N'-desmethyltamoxifen hydrochloride, α-hydroxy-N-desmethyltamoxifen, N,N'-desmethylytamoxifen hydrochloride, 3-hydroxytamoxifen citrate, 4-hydroxytamoxifen or the solvent alone. Twenty-four hours following the last treatment, the rats were killed. The data are expressed as the mean ± SD.

Significantly different (P < 0.05) from rats treated with solvent alone as determined by one-way ANOVA followed by Dunnett’s test.

Table I. Body weight changes and liver and uterus weights in female Sprague–Dawley rats treated by gavage or intraperitoneally with seven daily doses of tamoxifen and its derivatives*  

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body weight change (g)</th>
<th>Liver (g)</th>
<th>Uterus (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gavage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solvent</td>
<td>29 ± 8</td>
<td>11.45 ± 1.64</td>
<td>378 ± 45</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>7 ± 8</td>
<td>11.67 ± 0.95</td>
<td>299 ± 48</td>
</tr>
<tr>
<td>α-Hydroxytamoxifen</td>
<td>7 ± 8</td>
<td>13.47 ± 1.10</td>
<td>297 ± 43</td>
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<tr>
<td>N-Desmethyltamoxifen</td>
<td>15 ± 6b</td>
<td>11.00 ± 1.83</td>
<td>268 ± 35</td>
</tr>
<tr>
<td>α-Hydroxy-N-Desmethyltamoxifen</td>
<td>12 ± 5b</td>
<td>14.03 ± 0.86</td>
<td>311 ± 25</td>
</tr>
<tr>
<td>N,N'-Desmethyltamoxifen</td>
<td>13 ± 6b</td>
<td>10.66 ± 1.30</td>
<td>324 ± 67</td>
</tr>
<tr>
<td>Intraperitoneal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solvent</td>
<td>−1 ± 6</td>
<td>8.47 ± 1.15</td>
<td>375 ± 51</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>−13 ± 5b</td>
<td>8.44 ± 1.06</td>
<td>241 ± 29b</td>
</tr>
<tr>
<td>α-Hydroxytamoxifen</td>
<td>−14 ± 5b</td>
<td>10.17 ± 1.2b</td>
<td>249 ± 23b</td>
</tr>
<tr>
<td>3-Hydroxytamoxifen</td>
<td>−22 ± 5b</td>
<td>8.05 ± 0.84</td>
<td>243 ± 25b</td>
</tr>
<tr>
<td>4-Hydroxytamoxifen</td>
<td>−12 ± 5b</td>
<td>9.24 ± 1.37</td>
<td>276 ± 32b</td>
</tr>
</tbody>
</table>

*Female Sprague–Dawley rats were treated by gavage or intraperitoneal injection with seven daily doses of 54 μmol/kg tamoxifen. Additional animals were treated with equimolar doses of α-hydroxytamoxifen, α-hydroxy-N-desmethyl tamoxifen, N,N'-desmethyl tamoxifen hydrochloride, α-hydroxy-N-desmethyl tamoxifen, N,N'-desmethylytamoxifen hydrochloride, 3-hydroxytamoxifen citrate, 4-hydroxytamoxifen or the solvent alone. Twenty-four hours following the last treatment, the rats were killed. The data are expressed as the mean ± SD.

Significantly different (P < 0.05) from rats treated with solvent alone as determined by one-way ANOVA followed by Dunnett’s test.

**Post-labeling analyses**

Following injection of unbound materials and precipitation, the modified DNA was hydrolyzed to nucleosides as described above. HPLC analysis indicated one major adduct, which has been characterized as (E)-α-(deoxyguanosin-Ν²-yl)-4-hydroxytamoxifen (38), were detected by HPLC.

Additional animals were treated in the same manner with equimolar doses of α-hydroxytamoxifen (12 rats; 20.9 mg/kg), α-hydroxy-N-desmethyltamoxifen (12 rats; 21.2 mg/kg), α-hydroxy-N,N'-desmethyl tamoxifen (4 rats; 20.1 mg/kg), N,N'-desmethyl tamoxifen hydrochloride (4 rats; 20.5 mg/kg) or the solvent alone (12 rats; 200 μl trioctanoin). Two additional rats, which served as positive controls for the mutation analyses, were given a single intraperitoneal injection of 150 mg/kg N-ethyl-N-nitrosourea (ENU; Sigma), administered in 2 ml phosphate-buffered saline.

Bone marrow was aspirated from the femurs and humeri. Hepatic nuclei characterized by comparison with the DNA adduct standards modified in vitro with α-acetoxytamoxifen, α-sulfoxide-N-desmethyltamoxifen and 4-hydroxytamoxifen quinone methide. The adduct levels were quantified through comparison to a 3P-post-labeled DNA adduct standard containing (E)-α-(deoxyguanosin-Ν²-yl)tamoxifen at a level of 5.2 adducts/10⁶ nucleotides.

Lymphocyte Hprt mutant assay

The lymphocyte Hprt mutant assay was performed as described previously (54,55). Briefly, spleens were removed aseptically from rats, teased apart with 25–28 gauge needles and washed with cold, supplemented phosphate-buffered saline to release the cells. Lymphocytes were isolated by Accuc-Paque (Accurate Chemical, Westbury, NY) density-gradient centrifugation. For each sample, two sets of lymphocyte cultures were established in three 96-well microtiter plates. One set of plates was used for determining the cloning efficiency under...
Tamoxifen DNA adducts

Fig. 3. Persistence of liver DNA adducts. DNA adduct levels were assessed 1 and 3 months after the last dose in rats given tamoxifen (Tam), α-hydroxytamoxifen (α-OH-Tam) or N-desmethyltamoxifen (DeMeTam) by gavage. The data are presented as the mean ± SD from four rats relative to the values obtained from animals killed 1 day after the last dose.

Fig. 2. HPLC of liver DNA from female Sprague–Dawley rats administered orally (A) solvent, (B) tamoxifen, (C) α-hydroxytamoxifen, (D) N-desmethyltamoxifen or (E) α-hydroxy-N-desmethyltamoxifen, and DNA modified in vitro with (F) α-acetoxytamoxifen or (G) α-sulfoxy-N-desmethyltamoxifen. The 32P-post-labeling and elution conditions are outlined in Materials and methods.

Table II. DNA adduct levels in liver DNA of female Sprague–Dawley rats treated by gavage or intraperitoneally with seven daily doses of tamoxifen and its derivatives

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hepatic DNA adduct levels (adducts/10^7 nucleotides)</th>
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</thead>
<tbody>
<tr>
<td>Gavage</td>
<td></td>
</tr>
<tr>
<td>Solvent</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>84.4 ± 46.9^b</td>
</tr>
<tr>
<td>α-Hydroxytamoxifen</td>
<td>187.6 ± 22.9^b</td>
</tr>
<tr>
<td>N-Desmethyltamoxifen</td>
<td>113.1 ± 19.2^b</td>
</tr>
<tr>
<td>α-Hydroxy-N-desmethyltamoxifen</td>
<td>69.4 ± 23.3^b</td>
</tr>
<tr>
<td>N,N-Didesmethyltamoxifen</td>
<td>0.9 ± 0.9</td>
</tr>
<tr>
<td>Intraperitoneal</td>
<td></td>
</tr>
<tr>
<td>Solvent</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>153.8 ± 52.7^b</td>
</tr>
<tr>
<td>α-Hydroxytamoxifen</td>
<td>749.4 ± 140.7^b</td>
</tr>
<tr>
<td>3-Hydroxytamoxifen</td>
<td>10.7 ± 12.1</td>
</tr>
<tr>
<td>4-Hydroxytamoxifen</td>
<td>3.6 ± 1.8</td>
</tr>
</tbody>
</table>

^aFemale Sprague–Dawley rats were treated by gavage or intraperitoneal injection with seven daily doses of 54 µmol/kg tamoxifen. Additional animals were treated with equimolar doses of α-hydroxytamoxifen, N-desmethyltamoxifen hydrochloride, α-hydroxy-N-desmethyltamoxifen, N,N-didesmethyltamoxifen hydrochloride, 3-hydroxytamoxifen citrate, 4-hydroxytamoxifen or the solvent alone. Twenty-four hours following the last treatment, the rats were killed. The data are expressed as the mean ± SD. The limit of detection was ~3 adducts/10^8 nucleotides.

^bSignificantly different (P < 0.05) from rats treated with solvent alone as administered intraperitoneally (Table I).

Results

Body and organ weight changes

Female Sprague–Dawley rats were treated for 7 days by gavage or intraperitoneal injection with tamoxifen and a number of its derivatives. Compared with rats treated with the solvent alone, each compound caused a significant reduction in body weight when measured 1 day after the last treatment (Table I). Liver weight was not affected, except by α-hydroxytamoxifen administered intraperitoneally (Table I). Uterine weight tended to decrease, but the change was only significant with the derivatives given intraperitoneally (Table I). Spleen and thymus weights were also measured in rats dosed by gavage; these did not differ from the solvent-treated control rats (data not shown). Additional rats were administered tamoxifen, α-hydroxytamoxifen and N-desmethyltamoxifen by gavage for 7 days and killed 1 and 3 months after the last dose. With non-selective conditions, and the other was supplemented with 2.5 µg/ml 6-thioguanine and used to select 6-thioguanine-resistant lymphocytes. Both sets of plates were incubated in a humidified atmosphere of 5% CO₂ in air and after 11 days the plates were scored for clone formation (56). The cloning efficiency for each set of cultures and the frequency of 6-thioguanine-resistant lymphocytes were then calculated.
these rats, there were no significant differences in body or organ weights compared with the controls.

**DNA adduct analyses**

DNA adducts were assessed by $^{32}$P-post-labeling in conjunction with HPLC. When measured 1 day after the last treatment, tamoxifen (Figure 2B) and $\alpha$-hydroxytamoxifen (Figure 2C) gave two major adducts (b and c) and one minor adduct (a) in the liver DNA from rats treated by gavage. These adducts were not observed in control rats (Figure 2A). A similar pattern was observed in rats administered tamoxifen and $\alpha$-hydroxytamoxifen intraperitoneally (data not shown). One major adduct (b) and one minor adduct (a) were detected in liver DNA from rats dosed with $N$-desmethyltamoxifen (Figure 2D) and $\alpha$-hydroxy-$N$-desmethyltamoxifen (Figure 2E) by gavage. Adduct b co-chromatographed with $(E)\alpha$-(deoxyguanosin-$N^2$-yl)-$N$-desmethyltamoxifen (Figure 2G), while adduct c co-chromatographed with $(E)\alpha$-(deoxyguanosin-$N^2$-yl)tamoxifen (Figure 2F). Only background levels of DNA adducts were detected in liver DNA from rats dosed by gavage with $N$,$N$-didesmethyltamoxifen or injected intraperitoneally with 3-hydroxytamoxifen or 4-hydroxytamoxifen (data not shown). Similarly, none of the compounds resulted in an increase in DNA adducts in uterus, spleen, thymus or bone marrow DNA from rats treated by gavage or in uterus DNA from rats injected intraperitoneally (data not shown). Table II presents a summary of the binding data for liver DNA. When assessed 1 day following the last dose, the relative levels of binding in rats treated by gavage were $\alpha$-hydroxytamoxifen > tamoxifen ≈ $N$-desmethyltamoxifen ≈ $\alpha$-hydroxy-$N$-desmethyltamoxifen > $N$,$N$-didesmethyltamoxifen. In rats dosed intraperitoneally, the relative order of binding was $\alpha$-hydroxytamoxifen > tamoxifen > 3-hydroxytamoxifen ≈ 4-hydroxytamoxifen.

Hepatic DNA binding levels were also assessed 1 and 3 months after the last dose in rats administered tamoxifen, $\alpha$-hydroxytamoxifen and $N$-desmethyltamoxifen by gavage. When assayed 1 month after treatment, the adduct levels had decreased to 14–37% of the values obtained 1 day after the last dose (Figure 3). These values decreased to 6–9% of the initial levels when measured 3 months after dosing.

**Hprt mutant analysis**

One and 3 months after the last treatment, rats administered tamoxifen and $\alpha$-hydroxytamoxifen were killed to assess the extent of mutant induction in spleen T-lymphocytes. Compared with rats administered the solvent alone, there was no significant increase in mutants (Figure 4). A substantial increase in mutant frequency was observed with ENU, which served as a positive control.

**Table III. Hepatic and uterine enzyme activities in female Sprague–Dawley rats treated intraperitoneally with seven daily doses of tamoxifen, $\alpha$-hydroxytamoxifen, 3-hydroxytamoxifen or 4-hydroxytamoxifen**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hepatic Activity</th>
<th>Uterine peroxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ethoxyresorufin $O$-deethylation</td>
<td>$p$-Nitrophenol hydroxylation</td>
</tr>
<tr>
<td>Solvent</td>
<td>8.3 ± 4.7</td>
<td>129 ± 43</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>19.4 ± 2.6$^{b}$</td>
<td>128 ± 65</td>
</tr>
<tr>
<td>$\alpha$-Hydroxytamoxifen</td>
<td>22.7 ± 5.6$^{b}$</td>
<td>194 ± 66</td>
</tr>
<tr>
<td>3-Hydroxytamoxifen</td>
<td>10.1 ± 4.5</td>
<td>88 ± 52</td>
</tr>
<tr>
<td>4-Hydroxytamoxifen</td>
<td>16.5 ± 5.8$^{b}$</td>
<td>107 ± 27</td>
</tr>
</tbody>
</table>

$^{a}$Female Sprague–Dawley rats were treated by intraperitoneal injection with seven daily doses of 54 $\mu$mol/kg tamoxifen. Additional animals were treated with equimolar doses of $\alpha$-hydroxytamoxifen, 3-hydroxytamoxifen citrate, 4-hydroxytamoxifen or the solvent alone. Twenty-four hours following the last treatment, the rats were killed. The data are presented as the mean ± SD. Hepatic enzyme activities are expressed in pmol/mg protein/min; uterine peroxidase activity is given as mU/mg protein.

$^{b}$Significantly different ($P < 0.05$) from rats treated with solvent alone as determined by one-way ANOVA followed by Dunnett’s test.

**Discussion**

An increased incidence of endometrial cancer has been associated with the use of tamoxifen as either an adjuvant chemotherapeutic or a chemopreventive agent for breast cancer (1,2). The mechanism for the induction of these tumors is not known, although support for a genotoxic pathway comes from the observation of DNA adducts in endometrial samples from women treated with tamoxifen (57–59). Other investigators, however, have not detected an increase in endometrial DNA adducts (60,61) and it has been suggested that the adducts that have been detected may not arise from tamoxifen (62).

Tamoxifen is also carcinogenic in experimental animals (3–10). In rats, tumors occur in the liver (3–5) and uterus (6,7,10), with the hepatic tumors being ascribed to a genotoxic mechanism resulting from the formation of tamoxifen DNA adducts (4,11–15). Since the mechanism for the induction of uterine tumors in any species is not known, we have used the rat model to investigate if a genotoxic pathway could be responsible for the endometrial tumors. In order to maximize the possibility of adduct detection, the animals were also...
treated with tamoxifen derivatives that have been proposed to be proximate carcinogens in the metabolic activation of this anti-estrogen.

DNA adducts were readily detected in hepatic DNA after treating rats with tamoxifen by either gavage or intraperitoneal injection. The adduct levels did not differ between the two routes of administration but are ~10-fold higher than we previously reported after intraperitoneal dosing (39). In the present study, the adduct levels were quantified through comparison to a DNA standard that had been modified with tamoxifen at a known level, whereas previously (39) the levels were estimated based upon the extent of 32P incorporation and the specific activity of the [γ-32P]ATP used for 32P-post-labeling. These results indicate that the efficiency of labeling tamoxifen DNA adducts is ~10%, which emphasizes the importance of having well characterized DNA standards for DNA adduct quantification by 32P-post-labeling. A similar finding was reported by us when assessing DNA adduct levels from the carcinogen 4-aminobiphenyl (63). Other investigators, however, have reported a considerably higher 32P-post-labeling efficiency for tamoxifen DNA adducts when using relative adduct labeling as the method of quantitation (30).

Three DNA adducts were detected in liver DNA from rats treated with tamoxifen. As previously reported (25,28,31,32), two of these arise from α-hydroxylation of tamoxifen (adduct c, Figure 2B) and N-desmethyltamoxifen (adduct b, Figure 2B). The identity of the third adduct (adduct a, Figure 2B) is not known. It does not appear to result from N,N-didesmethyltamoxifen because this metabolite gave only very low binding to hepatic DNA (Table II). A similar low level of hepatic DNA binding by N,N-didesmethyltamoxifen has been reported by Brown et al. (31). Considering the structural similarity of tamoxifen, N-desmethyltamoxifen and N,N-didesmethyltamoxifen, it is likely that the α-hydroxylation of N,N-didesmethyltamoxifen and subsequent esterification occurs in vivo. It is conceivable that the presence of a primary amino group in N,N-didesmethyltamoxifen may allow an efficient conjugation and excretion. However, adduct a could still arise from α-hydroxy-N,N-didesmethyltamoxifen, formed by an alternative pathway involving N-deethylation of α-hydroxy-N-desmethyltamoxifen, as suggested by Phillips et al. (30). Further experiments will be necessary to test this possibility.

When tamoxifen and α-hydroxytamoxifen were administered by intraperitoneal injection, α-hydroxytamoxifen bound to hepatic DNA to a 5-fold greater extent than was found for tamoxifen (Table II). This difference is similar, although of a lower magnitude, to what has been reported previously in F344 rats treated intraperitoneally (18). When administered by gavage, the binding of α-hydroxytamoxifen to hepatic DNA was only 2-fold greater than that observed with tamoxifen (Table II) and the binding of α-hydroxy-N-desmethyltamoxifen did not exceed that found with N-desmethyltamoxifen (Table II). These results were unexpected because in F344 rats, α-hydroxytamoxifen gave 30-fold higher hepatic DNA adduct levels than tamoxifen when administered by gavage (30). The relatively small difference in binding of the α-hydroxy derivatives compared with tamoxifen after gavage dosing in the current experiments may be due to the use of Sprague–Dawley rats or the multiple dose sequence. In addition, a comparison of the results obtained after intraperitoneal versus gavage treatment indicates that decomposition of the α-hydroxy derivatives may occur when administered by gavage due to protonation of the hydroxyl functions in the acidic environment of the stomach.

Although extensive hepatic DNA adduct formation occurred with tamoxifen, N-desmethyltamoxifen and their α-hydroxy metabolites, binding was not detected in other tissues. Two major pathways have been proposed for the metabolic activation of tamoxifen: sulfotransferase-catalyzed sulfation of α-hydroxytamoxifen and α-hydroxy-N-desmethyltamoxifen (20–22,24) and peroxidase-catalyzed oxidation of 4-hydroxytamoxifen (34–37). In the rat, the sulfation of the α-hydroxy metabolites is catalyzed by hydroxysteroid sulfotransferase a (rHSTa; 21). rHSTa is found at high levels in rat liver, but is expressed at very low levels in non-hepatic tissues (64–66). α-Hydroxytamoxifen has been detected in the bile of rats administered tamoxifen (67) and the weight changes we observed in the uterus (Table I) suggest that the administered compounds were distributed systemically. The failure to detect DNA adducts derived from α-hydroxytamoxifen or α-hydroxy-N-desmethyltamoxifen in the uterus or other non-hepatic tissues is consistent with the low levels of rHSTa in extrahepatic tissues. It also suggests that any α-sulfoxyl derivatives formed in the liver are not sufficiently stable to be transported systemically.

Pathak et al. (37) reported that intraperitoneal administration of tamoxifen increased uterine peroxidase activity 10-fold, which could result in an increased oxidation of 4-hydroxytamoxifen to 4-hydroxytamoxifen quinone methide. We were unable to confirm this observation; instead of an increase in uterine peroxidase activity, we detected a 2-fold decrease after intraperitoneal administration of tamoxifen and a number of its derivatives (Table III), which is very similar to what we previously found after treating rats with tamoxifen by gavage (39). This decrease in peroxidase activity is consistent with the anti-estrogenicity of tamoxifen for the rat uterus (68). We also obtained no evidence for DNA adducts being formed from 4-hydroxytamoxifen quinone methide in either the liver or uterus. Fan et al. (69) have suggested that 4-hydroxytamoxifen quinone methide has unusual stability; nonetheless, even if this metabolite is formed in vivo, it either does not have sufficient reactivity or is trapped by other nucleophiles before reacting with DNA.

Although we did not detect DNA adducts indicative of α-hydroxytamoxifen, α-hydroxy-N-desmethyltamoxifen or 4-hydroxytamoxifen in non-hepatic tissues, it is possible that DNA damage was occurring that was not detected by the 32P-post-labeling analyses. For example, tamoxifen and certain of its metabolites have been proposed to lead to oxidative DNA damage (e.g. 8-oxodeoxyguanosine; 70,71), a type of DNA adduct that would not be detected by our methodology. To evaluate the possibility of other types of DNA damage being formed extrahepatically, we assessed the mutant frequency in the Hprt gene of spleen lymphocytes from rats treated with tamoxifen and α-hydroxytamoxifen. When measured 1 and 3 months after the last dose, no increase in the mutant frequency was observed (Figure 4), thus supporting the conclusion that tamoxifen and its metabolites are not genotoxic extrahepatically. As part of the mutagenesis experiment, hepatic DNA adduct levels were also determined and found to have decreased to 6–9% their initial levels 3 months after the last dose (Figure 3). This decrease in adduct levels is consistent with what has been reported by Divi et al. (72) using an immunochemical approach.
In conclusion, these experiments confirm previous observations that tamoxifen is activated to a genotoxic agent in rat liver through \( \alpha \)-hydroxylation. We found no evidence for DNA adduct formation through a quinone methide pathway and, furthermore, found no evidence for tamoxifen genotoxicity in non-hepatic tissues. Since tamoxifen is tumorigenic in endometrial tissue in rats, our results suggest that these tumors do not arise from the formation of tamoxifen–DNA adducts. This implies that tamoxifen analogues designed to be less genotoxic than tamoxifen (e.g. toremifene) but which retain a similar estrogen/anti-estrogen profile may pose a risk for endometrial tissue.

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