Comparison of the DNA adducts formed by tamoxifen and 4-hydroxytamoxifen in vivo

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Tamoxifen is a liver carcinogen in rats and has been associated with an increased risk of endometrial cancer in women. Recent reports of DNA adducts in leukocyte and endometrial samples from women treated with tamoxifen suggest that it may be genotoxic to humans. One of the proposed pathways for the metabolic activation of tamoxifen involves oxidation to 4-hydroxytamoxifen, which may be further oxidized to an electrophilic quinone methide (18–22; Figure 1, pathway B). We have now compared the extent of DNA adduct formation in female Sprague–Dawley rats treated by gavage with tamoxifen and 4-hydroxytamoxifen. We have also assessed the effect of tamoxifen and 4-hydroxytamoxifen upon liver and uterine weights and microsomal enzyme activities.

Materials and methods

Instrumentation

Reversed phase HPLC analyses were conducted using a μBondapak C18 column (0.39×30 cm; Waters Associates, Milford, MA) on an HPLC system consisting of two Waters Model 510 pumps, a Rheodyne Model 7125 injector (Rheodyne, Cotati, CA) and a Waters Model 660 automated gradient controller. The peaks were monitored at 280 nm with a Hewlett-Packard 1050 diode array spectrophotometric detector (Hewlett-Packard, Wilmington, DE). When conducting 32P-post-labeling analyses by HPLC, 32P was monitored with a Radiomatic Flu-One Model A-500 on-line radioactivity detector (Packard Instruments, Meriden, CT).

Chemicals

Tamoxifen, salmon testes DNA, bis[2-hydroxyethyl]iminotris(hydroxymethyl)methane (Bis-Tris), tricaprylin, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, Folin and Ciocalteu’s phenol reagent, micrococal nuclease, spleen phosphodiesterase, nuclease P1, potato apyrase and snake venom phosphodiesterase were purchased from Sigma (St Louis, MO), T4 polynucleotide kinase, spleen phosphodiesterase, nuclease P1, potato apyrase and horseradish peroxidase were purchased from ICN Pharmaceutical (Irvine, CA), p-Nitrophenol, p-nitrocatechol, 4-dimethylaminopyrine and 4-aminooantipyrine were acquired from Aldrich (Milwaukee, WI).

4-Hydroxytamoxifen was obtained as a mixture (~1:1) of the cis and trans isomers by condensation of 4-hydroxy-4′-(2-dimethylamino)ethoxy)benzo-phenone with the α-carbon anion from propylbenzene, followed by dehydration of the resulting diastereomeric carbinalons (24). 4-Hydroxytamoxifen quinone methide was prepared by oxidation of cis/trans-4-hydroxytamoxifen with an excess of freshly prepared active manganese dioxide (23). α-Hydroxytamoxifen was synthesized by the method of Foster et al. (25) and converted to α-acetoxytamoxifen as described by Osborne et al. (26).

DNA standards

An acetone solution containing 4-hydroxytamoxifen quinone methide was added to salmon testes DNA that had been dissolved in 5 mM Bis-Tris, 0.1 mM EDTA, pH 7.1 (23). The mixture was protected from light and incubated overnight at 37°C. Following evaporation of the organic solvent, decomposition products were extracted sequentially with diethyl ether and n-butanol, both of which had been preextracted with 5 mM Bis-Tris, 0.1 mM EDTA, pH 7.1, and the 4-hydroxytamoxifen-modified DNA was precipitated with NaCl and ethanol. The modified DNA was dissolved in 5 mM Bis-Tris, 0.1 mM EDTA, pH 7.1, and an aliquot was hydrolyzed with DNase I, alkaline phosphatase and snake venom phosphodiesterase (23). Analysis by HPLC indicated two major adducts that have been identified as (E)- and (Z)-α-deoxyguanosino-N2-yl)-4-hydroxytamoxifen (23).

α-Acetoxytamoxifen was reacted with DNA by the procedure of Osborne et al. (26) and the modified DNA was purified as described above. Enzymatic hydrolysis followed by HPLC analysis indicated a profile consisting of one

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Introduction

Tamoxifen is an important adjunct chemotherapeutic agent for treating women with breast cancer (1). This non-steroidal antiestrogen has also been shown to decrease breast cancer incidence in healthy women at high risk for the disease (2). In spite of these benefits, tamoxifen is known to be a liver carcinogen in rats (3–5) and has been found to increase the risk of endometrial cancer in women (6–12; reviewed in refs 13,14). In addition, the occurrence of DNA adducts in leukocyte and endometrial samples from women treated with tamoxifen suggests that it may be genotoxic to humans (15–17).

One of the proposed pathways for the metabolic activation of tamoxifen involves oxidation to 4-hydroxytamoxifen, which may be further oxidized to an electrophilic quinone methide (18–22; Figure 1, pathway B). We have recently shown that 4-hydroxytamoxifen quinone methide reacts with DNA to form covalent adducts (23). The major products, which resulted from 1,8-addition of the exocyclic nitrogen of deoxyguanosine to the conjugated system of 4-hydroxytamoxifen quinone methide, were characterized as (E)- and (Z)-α-deoxyguanosino-N2-yl)-4-hydroxytamoxifen (23). We have now compared the extent of DNA adduct formation in female Sprague–Dawley rats treated by gavage with tamoxifen and 4-hydroxytamoxifen.
Fig. 1. Proposed metabolic activation pathways for tamoxifen. Only the major DNA adducts resulting from α-hydroxytamoxifen and 4-hydroxytamoxifen quinone methide are shown.

major and three minor adducts that had UV spectra consistent with those reported by Osborne et al. (26,27). The major adduct has been identified as (E)-α-(deoxyguanosin-N2-yl)-tamoxifen (26), while the minor adducts have been characterized as diasteroisomers of (Z)-α-(deoxyguanosin-N2-yl)-tamoxifen and α-(deoxyadenosin-N6-yl)-tamoxifen (27).

Reaction of DNA with 4-hydroxytamoxifen in the presence of horseradish peroxidase

4-Hydroxytamoxifen (5 mg) was incubated with 8 mg of salmon testes DNA, 116 U of horseradish peroxidase and 20 µl of 30% hydrogen peroxide in 8 ml of 5 mM Bis-Tris, 0.1 mM EDTA, pH 7.1. The mixture was protected from light and incubated overnight at 37°C. The decomposition products were extracted and the modified DNA was precipitated as described above.

Treatment of animals

Rats were treated according to the protocol of White et al. (28). Specifically, eight female Sprague–Dawley rats [Crl:COBS CD (SD) BR outbred, 8 weeks old, 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 µm mol/kg, dissolved in 200 µl tricaprylin). Eight additional rats were treated daily for 7 days with 4-hydroxytamoxifen (21 mg/kg, 54 µm mol/kg, dissolved in 200 µl tricaprylin) and eight control animals were treated with 200 µl tricaprylin alone. Twenty-four hours following the last treatment, the rats were killed by exposure to carbon dioxide. Half of the animals were used to assess the induction of hepatic cytochrome P-450 and uterine peroxidase activities. The remaining rats were used to determine DNA adduct formation in vivo.

Hepatic microsomes were prepared by differential centrifugation (29). Uteri were removed as described by Lyttle and DeSombre (30) and uterine extracts were obtained in 250 mM sucrose, 500 mM CaCl2, according to the procedure of Pathak et al. (22). Protein content was determined by the Lowry procedure (31), using bovine serum albumin as the standard. To assess in vivo DNA adduct formation, hepatic nuclei were isolated by the method of Basler et al. (32) and DNA was prepared from the nuclei and whole uteri by slight modifications of the method described in Beland et al. (33).

32P-post-labeling analyses

32P-post-labeling analyses were conducted by the nuclease P1 enrichment procedure of Reddy and Randerath (34). Briefly, 10 µg DNA was hydrolyzed with micrococal endonuclease and spleen phosphodiesterase for 3 h at 37°C and then treated for an additional 1 h with nuclease P1. The samples were evaporated in a Speed-Vac concentrator and resuspended in water for labeling with 20 µCi carrier-free [γ-32P]ATP in the presence of T4 polynucleotide kinase. The incubation volume was 20 µl. In preliminary experiments, apyrase was added at the end of the incubation with T4 polynucleotide kinase to destroy excess [γ-32P]ATP. This did not improve the analyses. Likewise, incubations were also conducted with 2 µCi carrier-free [γ-32P]ATP and an incubation volume of 5 µl. Again, the analyses were not improved. Each analysis included DNA standards prepared from reactions with α-acetoxytamoxifen and 4-hydroxytamoxifen quinone methide.

Aliquots of the labeling mixture were analyzed by HPLC, which was conducted by a modification of the method of Möller and Zeisig (35). Specifically, adducts were separated on a 5 µ DeltaPak C18-100 column (3.9×150 mm; Waters Associates) using a gradient of 100% solvent A for 5 min, followed by a 35 min linear gradient to 100% solvent B and then an isocratic elution with solvent B for 20 min. Solvent A was 1.2 M ammonium...
Table I. Liver and uterus weights in female Sprague–Dawley rats treated with tamoxifen or 4-hydroxytamoxifen

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Treatment</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>Solvent</td>
<td>8.01 ± 0.333</td>
</tr>
<tr>
<td></td>
<td>Tamoxifen</td>
<td>7.76 ± 0.291</td>
</tr>
<tr>
<td></td>
<td>4-Hydroxytamoxifen</td>
<td>8.44 ± 0.269</td>
</tr>
<tr>
<td>Uterus</td>
<td>Solvent</td>
<td>0.38 ± 0.021</td>
</tr>
<tr>
<td></td>
<td>Tamoxifen</td>
<td>0.27 ± 0.006</td>
</tr>
<tr>
<td></td>
<td>4-Hydroxytamoxifen</td>
<td>0.29 ± 0.009</td>
</tr>
</tbody>
</table>

*Female Sprague–Dawley rats (8 weeks old, 8 per group) were treated by gavage with seven daily doses of tamoxifen (20 mg/kg, 54 µmol/kg, dissolved in 200 µl tricaprylin), 4-hydroxytamoxifen (21 mg/kg, 54 µmol/kg, dissolved in 200 µl tricaprylin) or 200 µl tricaprylin alone. Twenty-four hours following the last treatment, the rats were killed. The data are reported as means ± SEM.

1Significantly different (P < 0.05) from animals treated with the solvent alone as determined by one-way ANOVA followed by the Student–Newman–Keuls method for multiple pairwise comparisons.

formate, 10 mM ammonium phosphate, pH 4.5; solvent B was 24% acetonitrile in 1.2 M ammonium formate, 10 mM ammonium phosphate, pH 4.5. The flow rate was 1 ml/min.

Results and discussion

Organ weight changes in rats administered tamoxifen or 4-hydroxytamoxifen

Female Sprague–Dawley rats were treated by gavage daily for 7 days with tamoxifen, 4-hydroxytamoxifen or the solvent alone. When assessed 24 h after the last treatment, there were no significant differences in liver weights between any of the treatment groups (Table I); however, in rats treated with tamoxifen and 4-hydroxytamoxifen, the uterine weights were decreased by 25% (P < 0.05) compared with the solvent control group.

4-Hydroxytamoxifen is ~100-fold more potent than tamoxifen as an antiestrogen (42,43), with most of this activity being attributed to the trans-isomer [i.e. (Z)-4-hydroxytamoxifen (42,43)]. The observed decrease in uterine weight with tamoxifen and 4-hydroxytamoxifen is consistent with their antiestrogenic activities (44,45). 4-Hydroxytamoxifen is a relatively minor metabolite of tamoxifen, with the major metabolic pathway being N-demethylation to N-desmethyltamoxifen (46,47). Since tamoxifen and 4-hydroxytamoxifen had nearly identical effects on uterine weight, this indicates that only a small proportion of the administered 4-hydroxytamoxifen reached the uterus.

DNA adduct analyses in rats administered tamoxifen or 4-hydroxytamoxifen

DNA adducts formed in vivo were assessed by 32P-post-labeling analyses in combination with HPLC. Three DNA adducts, with retention times of 44, 46 and 48 min (peaks 1–3) and an approximate ratio of 1:5:4, were detected in liver DNA from rats administered tamoxifen (Figure 2a). Liver DNA from rats administered 4-hydroxytamoxifen had two adducts (Figure 2b) that corresponded in retention time to the two major adducts detected in liver DNA from rats given tamoxifen. The adduct levels in the liver of 4-hydroxytamoxifen-treated rats were 20-fold lower than those in the tamoxifen-treated animals (Table II) and did not differ significantly from those observed in the control rats, which also had two adducts in the same region of the chromatogram (Figure 2c and Table II). Adduct levels in uterus DNA from rats treated with tamoxifen and 4-hydroxytamoxifen did not differ significantly from those detected in control rats (Table II).

The 48 min adduct peak (peak 3) detected in liver DNA (Figure 2a) had the same retention time as the major adduct detected from the in vitro reaction of α-acetoxytamoxifen with DNA (Figure 2e). This adduct has been identified as α-(deoxyguanosin-Ν²-yl)-tamoxifen (26,27,48; Figure 1, pathway A). The retention times of the two peaks eluting at 44 and 46 min (peaks 1 and 2) in liver DNA (Figure 2a) corresponded to those of the minor adducts observed with the α-acetoxytamoxifen DNA standard (Figure 2e). Since α-acetoxytamoxifen gave only one major adduct in vitro, it is presently unclear if the second major adduct (i.e. the 46 min peak, peak 2) observed in liver DNA resulted from a reactive ester of α-hydroxytamoxifen or from a reactive derivative of another tamoxifen metabolite.

In humans (49–53) and rats (46,47,54), the major metabolic pathway for tamoxifen is through N-demethylation to give N-desmethyltamoxifen (Figure 1, pathway C). For example, when assessed in breast cancer patients after daily dosing for up to 2.5 years, N-desmethyltamoxifen was found in plasma at approximately twice the concentration of tamoxifen (51). Similar results have been found in human tissues (52) and in rats chronically administered tamoxifen (46). In addition to being found at higher concentrations, N-desmethyltamoxifen has a longer half-life than tamoxifen (50). N-desmethyltamoxifen is further metabolized to a number of products, one of which is α-hydroxy-N-desmethyltamoxifen [Figure 1, pathway C (53,54)]. Given the similarity in structure between α-hydroxytamoxifen and α-hydroxy-N-desmethyltamoxifen and the high tissue concentrations of N-desmethyltamoxifen, it is plausible that a reactive metabolite of α-hydroxy-N-desmethyltamoxifen (e.g. α-acetoxy- or α-sulfoxo-N-desmethyltamoxifen) may be responsible for the second major adduct (peak 2) detected in rats treated with tamoxifen. Since submission of this paper, Rajaniemi et al. (55) have presented data in support of this hypothesis.

The contribution of 4-hydroxytamoxifen quinone methide to the hepatic DNA adducts is uncertain. The two peaks observed in the in vitro standard [i.e. (E)- and (Z)-α-(deoxyguanosin-Ν²-yl)-4-hydroxytamoxifen; Figure 2d] did co-elute with the 44 and 48 min peaks (peaks 1 and 3) observed in liver DNA (Figure 2a); however, the low level of hepatic DNA adducts with 4-hydroxytamoxifen (Figure 2b and Table II) indicates that 4-hydroxytamoxifen quinone methide may not be an important reactive intermediate in this tissue. Alternatively, it is possible that the low extent of binding was due to the poor absorption of 4-hydroxytamoxifen when given by gavage to rats. Recently, Hardcastle et al. (56) demonstrated that α,4-dihydroxytamoxifen, an oxidation product of 4-hydroxytamoxifen that may be formed...
in vivo in equilibrium with 4-hydroxytamoxifen quinone methide (57), reacts 12-fold better than α-hydroxytamoxifen with DNA at acidic pH. Although the structures of these adducts were not elucidated (56), they are likely to be identical to those generated from 4-hydroxytamoxifen quinone methide. Since these putative metabolites of 4-hydroxytamoxifen have the ability to react with DNA to a significant degree, the low extent of hepatic DNA binding that we detected with 4-hydroxytamoxifen indicates that this metabolic activation pathway is unlikely to make a major contribution to the adducts formed in vivo.

It should also be noted that a low level of adducts occurred in control liver (Figure 2c) with the same retention times as those detected in the livers of rats treated with tamoxifen (Figure 2a) and 4-hydroxytamoxifen (Figure 2b). Therefore, the hepatic DNA adducts detected following administration of tamoxifen and 4-hydroxytamoxifen may not arise from these compounds but rather may be due to these antiestrogens altering the formation of endogenous DNA adducts. Such a possibility has been suggested for the tamoxifen DNA adducts reported to be formed in human endometrial samples (58).

White et al. (28) have used ³²P-post-labeling analyses in combination with TLC to examine the DNA adducts in female F344/n rats treated by gavage daily for 7 days with tamoxifen at doses of 5–45 mg/kg. Multiple adducts were detected in liver DNA, with one adduct accounting for >80% of the total. At the same dose used in the current experiment (i.e. 20 mg/
kg), the total adduct level was ~75 adducts/10^8 nucleotides. Considering the difference in strains of rats and also the uncertainties and interlaboratory variations that occur with 32P-post-labeling analyses (59), this value is reasonably similar to the 170 adducts/10^8 nucleotides that we found (Table II). In contrast to the work of White et al. (28), who reported one major and multiple minor adducts, we detected two adducts of nearly equal intensity, accompanied by a third minor adduct, in the livers of rats administered tamoxifen (Figure 2a). This discrepancy may be due to differences in the chromatographic systems (i.e. TLC versus HPLC) because, in other work from the same laboratory (60), two major adducts were found in liver DNA from tamoxifen-treated rats when the 32P-post-labeling analyses were conducted using HPLC.

White et al. (28) did not detect binding to uterus DNA, even at a tamoxifen dose of 45 mg/kg. In contrast, uterine DNA adducts have been detected by Pathak et al. (22) in Sprague-Dawley rats treated with seven 20 mg/kg i.p. injections of tamoxifen. In their work, the binding to uterus DNA was ~37-fold lower than that found in the liver, which is nearly identical to the 35-fold difference that we detected (Table II). Although Pathak et al. (22) found multiple adducts in the liver, only one adduct was detected in the uterus; this adduct also appeared to be in the liver, but accounted for only a very minor amount (~3%) of the total binding. Through comparison with in vitro experiments, they suggested that this adduct arose from peroxidase activation of 4-hydroxytamoxifen. A similar metabolic activation pathway has been proposed to occur in the livers of mice treated with tamoxifen (21). While our observations are consistent with a relatively minor role of 4-hydroxytamoxifen activation in rat liver (vide supra), our experiments to date do not allow us to draw definite conclusions concerning activation pathways in the uterus. When we incubated 4-hydroxytamoxifen with DNA in vitro in the presence of horseradish peroxidase, we found an adduct profile by HPLC (data not shown) that was virtually identical to the one obtained from 4-hydroxytamoxifen quinone methide. This observation indicates that peroxidases have the ability to mediate the oxidation of 4-hydroxytamoxifen to a DNA-binding electrophile. This activation pathway may not be important in rat uterus because the adduct levels in the uteri of the tamoxifen- and 4-hydroxytamoxifen-treated rats did not differ from controls.

**Induction of hepatic and uterine enzyme activities**

To determine if repeated oral dosing with tamoxifen or 4-hydroxytamoxifen affected cytochrome P-450 activities, hepatic microsomal preparations were assayed for the induction of cytochrome P-450 1A1/1A2 (as measured by ethoxyresorufin deethylase activity), 2E1 (as measured by p-nitrophenol hydroxylase activity) and 3A4 (as measured by 4-dimethylaminopyrine demethylase activity). These assays were selected in view of studies indicating that the N-demethylation of tamoxifen to N-desmethytamoxifen is catalyzed by cytochrome P-450 1A1 (61) and members of the 3A family (62,63), while ring oxidation to give 4-hydroxytamoxifen appears to be catalyzed primarily by cytochrome P-450 2D6 (64) and to a lesser extent by cytochrome P-450 2E1 (65). In addition, incubation of tamoxifen with cell lines containing cytochrome P-450 2E1, 3A4 or 2D6 leads to genotoxicity, although the specific genotoxic metabolites were not identified (65). None of the activities was altered to a significant extent by treatment with either tamoxifen or 4-hydroxytamoxifen (data not shown). White et al. (66) did observe an ~2-fold induction in hepatic microsomal ethoxyresorufin deethylase activity in F344 rats treated daily by gavage with tamoxifen. Our failure to find induction of ethoxyresorufin deethylase activity could be due to the difference in rat strains or to the fact that White et al. (66) administered a 2.5-fold greater dose.

In addition to measuring hepatic microsomal activities, assays were conducted on uterine peroxidase activity. Treatment with tamoxifen or 4-hydroxytamoxifen decreased uterine peroxidase activity by ~50% (Table III), a variation that was marginally significant (P = 0.06 by one-way ANOVA). This result differs from that reported by Pathak et al. (22) who found a nearly 10-fold induction of peroxidase activity in Sprague-Dawley rats treated i.p. with tamoxifen. This difference in response could be due to the difference in the route of administration.

**Conclusions**

In this study, we confirm extensive hepatic DNA adduct formation by tamoxifen. As reported by others (26,67), an adduct resulting from α-hydroxytamoxifen is clearly present. This was accompanied by a second major adduct, the identity of which is currently unknown. DNA adducts could be detected in uterine DNA from rats treated with tamoxifen but the levels did not differ from those observed in control animals. Although sequential oxidation of tamoxifen to 4-hydroxytamoxifen and 4-hydroxytamoxifen quinone methide (and/or α,4-dihydroxytamoxifen) has been proposed as a potential metabolic activation pathway for tamoxifen, the low extent of DNA binding we detected with 4-hydroxytamoxifen suggests that this is not a major pathway. This observation is important in view of the fact that 4-hydroxytamoxifen is a potent antiestrogen currently undergoing clinical trials along with its 4-phosphate derivative (68).

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**References**


Tamoxifen and 4-hydroxytamoxifen DNA adducts


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