ACCELERATED PAPER

Activation of 4-hydroxytamoxifen and the tamoxifen derivative metabolite E by uterine peroxidase to form DNA adducts: Comparison with DNA adducts formed in the uterus of Sprague–Dawley rats treated with tamoxifen

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Introduction

Tamoxifen ([Z]-2-[4-(1,2-diphenyl-1-butenyl)-phenoxy]-N,N-dimethyl ethanamine) (TAM*), is an antiestrogenic compound used to treat breast cancer (1,2). In addition to its established chemotherapeutic use, it is being evaluated as a prophylactic agent in women at high risk for breast cancer (3). However, recent studies show that women treated with TAM have up to a 6-fold increased risk for uterine cancer (4–7). The biochemical processes associated with this increased risk have not been determined.

TAM is a strong liver carcinogen in rats (8–10) and a high frequency of p53 mutations are detected in hepatocarcinomas induced by TAM treatment (11). Although the mechanisms by which TAM produces both p53 mutations and liver carcinomas have not been determined, several laboratories (8,12–14) have reported that administration of TAM results in the formation of DNA adducts in the livers of rats, mice, and hamsters. These DNA adducts are likely to contribute to the mutagenic and carcinogenic effects of TAM (15).

In contrast to the above results with rodents, no evidence for the formation of TAM-DNA adducts in the liver of patients treated with TAM has been obtained (16). These results are in agreement with clinical studies which have also not demonstrated a significant increase in the frequency of liver cancer after TAM administration (7). In humans the carcinogenic effect of TAM appears to involve the uterine endometrium (4–7). These results suggest that, as observed with other carcinogens, initial metabolism of TAM may occur in the liver with subsequent accumulation of TAM metabolites in the susceptible tissues where they are further activated to produce their carcinogenic effect (17–19). Support for this hypothesis is derived from the observation that both 4-HO-TAM and N-desmethyl TAM have been shown to accumulate in the uterus of both humans and rodents after administration of TAM (20–24).

Peroxidase activity has been detected in uterine tissues of both rodents (25,26) and humans (27). In addition treatment with either estradiol or DES significantly increases the level of peroxidase activity in the uterus (25,26). Peroxidases such as HRP, lactoperoxidase and myeloperoxidase have been established to activate a variety of compounds to form DNA adducts (28–32). Recently we have demonstrated that HRP could activate both 4-HO-TAM and cis/trans-metabolite E to form DNA adducts (33,34). These results suggest that uterine peroxidase found in endometrial tissues may further activate TAM metabolites to form DNA adducts (Figure 1). These DNA adducts have been compared with those formed by HRP activation of these TAM metabolites.

Materials and methods

Chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI) and Sigma Chemical Co. (St Louis, MO). 4-HO-TAM was generously provided by Besin Iscovesco (Paris, France). cis/trans-Metabolite E was a 1:1 mixture of the cis and trans isomers was synthesized and characterized as previously described (34).

In vivo studies

Female Sprague–Dawley rats (8 weeks old, 165 g) (Charles River Laboratories, Hollister, CA) were divided into 4 groups each group consisting of 3–5
Activation of 4-HO-TAM and cis/trans-metabolite E by HRP described above. Reactions were carried out for 1 h at 37°C in a water bath. Control studies were performed without the addition of H$_2$O$_2$ to the incubation mixture, activation of 4-HO-TAM with uterine extracts (Table II) were similar to the results with 4-HO-TAM, the relative adduct levels produced by activation of cis/trans-metabolite E was 3.7-fold higher using the extracts prepared from uteri of rats treated with 20 mg/kg of TAM compared to extracts from the control treatment group (Table II). The relative adduct levels produced by activation of cis/trans-metabolite E using uterine extracts prepared from rats treated with varying doses of TAM. Without the addition of H$_2$O$_2$ to the reaction mixture, no DNA adducts were detected (data not shown). In contrast, daily i.p. injection of either 5, 10 or 20 mg/kg of TAM for 7 days produced a significant increase in the levels of peroxidase activity compared to the control group ($P < 0.001$). After treatment with 20 mg/kg of TAM for 7 days, a 10-fold increase in the level of peroxidase activity was found. The observed increase in peroxidase activity was correlated with the TAM treatment dose ($r^2 = 0.97, P = 0.01$).

4-HO-TAM was incubated with uterine extracts prepared from rats treated with varying doses of TAM. Without the addition of H$_2$O$_2$ to the reaction mixture, no DNA adducts were detected (data not shown). In contrast, daily i.p. injection of either 5, 10 or 20 mg/kg of TAM for 7 days produced a significant increase in the levels of peroxidase activity compared to the control group ($P < 0.001$). After treatment with 20 mg/kg of TAM for 7 days, a 10-fold increase in the level of peroxidase activity was found. The observed increase in peroxidase activity was correlated with the TAM treatment dose ($r^2 = 0.97, P = 0.01$).

Table I. Peroxidase activity in rat uteri after TAM treatment

<table>
<thead>
<tr>
<th>TAM (mg/kg)</th>
<th>Peroxidase activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.15 ± 0.02</td>
</tr>
<tr>
<td>5</td>
<td>0.35 ± 0.01</td>
</tr>
<tr>
<td>10</td>
<td>0.65 ± 0.13</td>
</tr>
<tr>
<td>20</td>
<td>1.52 ± 0.32</td>
</tr>
</tbody>
</table>

*Animals were treated daily for 7 days by i.p. injection with the doses indicated.

32P-postlabeling

The 32P nuclelease enhanced 32P-postlabeling technique was used as previously described for TAM-DNA adduct analysis (33,34,36). The DNA adducts were resolved on PEI-cellulose sheets using the following solvent systems: D$_2$O 1.0 M sodium phosphate pH 6.8; D$_2$O 3.0 M lithium formate and 7 M urea, pH 3.5; D$_2$O 0.72 M sodium phosphate, 0.46 M Tris and 7.6 M urea, pH 8.7 and D$_2$O 1.7 M sodium phosphate, pH 6. After chromatography, the adducts were located by autoradiography; scraped into liquid scintillation vials and counted. The relative adduct levels were calculated as previously described (33,34,36).

Rechromatography of 32P-postlabelled DNA adducts

The adducts were eluted from the PEI-cellulose plates with 0.5 ml of 1.5 M triethylammonium bicarbonate (pH 7.5). After evaporation to dryness, the samples were dissolved in 50 μl of water and spotted on one PEI-cellulose plate. The plates were developed in one direction in 0.72 M sodium phosphate, 0.46 M Tris and 7.6 M urea, pH 8.7.

Results

The levels of peroxidase activity in rat uteri were measured. Uteri from animals treated with tricaprylin had low levels of peroxidase activity (Table I). In contrast, daily i.p. injection of either 5, 10 or 20 mg/kg of TAM for 7 days produced a significant increase in the levels of peroxidase activity compared to the control group ($P < 0.001$). After treatment with 20 mg/kg of TAM for 7 days, a 10-fold increase in the level of peroxidase activity was found. The observed increase in peroxidase activity was correlated with the TAM treatment dose ($r^2 = 0.97, P = 0.01$).

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adducts were detected in rat uteri after 7 days treatment with TAM or HRP. (A) 4-HO-TAM activated by uterine extract, (B) cis/trans-metabolite E activated by uterine extract, (C) 4-HO-TAM activated by HRP, (D) cis/trans-metabolite E activated by HRP. The films were exposed at -70°C for 16 h, 16 h, 2 h and 4 h respectively.

![Image of DNA adducts](https://academic.oup.com/carcin/article-abstract/17/9/1785/337906)

Table II. Activation of 4-HO-TAM and cis/trans-metabolite E by uterine extract to form DNA adducts

<table>
<thead>
<tr>
<th>Uterine extract</th>
<th>DNA adduct levels × 10⁻⁸</th>
<th>Metabolite E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.5±0.24</td>
<td>0.4±0.01</td>
</tr>
<tr>
<td>5</td>
<td>2.5±1.10</td>
<td>0.7±0.1</td>
</tr>
<tr>
<td>10</td>
<td>3.3±0.50</td>
<td>1.0±0.2</td>
</tr>
<tr>
<td>20</td>
<td>5.5±0.24</td>
<td>1.5±0.3</td>
</tr>
</tbody>
</table>

formed by uterine extracts were significantly correlated with the levels of peroxidase activity ($r^2 = 0.95$, $P = 0.022$).

Activation of 4-HO-TAM and cis/trans-metabolite E to form DNA adducts was also investigated using the model peroxidase enzyme HRP. HRP activation of 4-HO-TAM produced one major DNA adduct (d) with a relative adduct level of 3.15 ± 0.6×10⁻⁷ (Figure 2D). HRP activation of cis/trans-metabolite E produced two major DNA adducts (d and e) with a relative adduct level of 3.15 ± 0.6×10⁻⁷ (Figure 2D).

The formation of DNA adducts in the liver and uterus of female Sprague-Dawley rats treated daily with 5 to 20 mg/kg of TAM for 7 days was investigated. No DNA adducts were detected in the tissues of the control group under these conditions (data not shown). The levels of DNA adducts produced by TAM treatment were dependent upon the treatment dose (Table III). Daily i.p. administration of TAM for 7 days produced a complex adduct pattern with 11 DNA adducts being detected (Figure 3A). The average distribution of the adducts were 1, 3.1%; 2, 5.7%; 3, 2.9%; 4, 2.1%; 5, 7.7%; 6, 50.4%; 7, 5.6%; 8, 4.3%; 9, 2.0%; 10, 17.6% and 11, 3.3%.

In contrast to the results observed in rat liver, no DNA adducts were detected in rat uteri after 7 days treatment with either 5 or 10 mg/kg of TAM (Figure 3B). However daily administration of 20 mg/kg of TAM for 1 week produced a single DNA adduct (1) with a relative adduct level of 0.64 ± 0.35×10⁻⁸ (Figure 3C).

Rechromatography experiments were conducted to compare the adducts formed by activation of 4-HO-TAM and cis/trans-metabolite E with either uterine extract or HRP with the DNA adducts formed in the uterus of animals treated with TAM. The result of this comparison suggests that adduct a produced by uterine extract activation of 4-HO-TAM was the same as adduct a produced by HRP activation of 4-HO-TAM (Figure 4A). In addition DNA adduct 1 detected in the uterus of rats treated with 20 mg/kg of TAM rechromatographed with adduct a formed by uterine extract activation of 4-HO-TAM (Figure 4A). Similar results were found after rechromatography of these adducts in two different solvent systems (data not shown). A similar analysis with adducts d and e produced by activation of cis/trans-metabolite E with either uterine extract or HRP suggests that they are the same (Figure 4B).

Discussion

A 7 day treatment with TAM significantly increased peroxidase activity in the rat uterus in a dose dependent manner. These results are consistent with TAM possessing weak estrogenic properties. Previous studies have demonstrated a significant increase in the level of uterine peroxidase activity following either single injections of rats with estradiol or DES (25,26) or multiple injections with TAM (21). In addition to this effect on peroxidase activity, related studies have demonstrated that administration of TAM increases the levels of several P450s including 2B1/2B2 and 3A1 (37,38); epoxide hydrolase and aldehyde dehydrogenase (39) and the levels of glutathione-S-transferase (39). These results demonstrate that administration of TAM produces pleiotropic effects in the rat.

Activation of 4-HO-TAM with uterine extract from TAM treated rats resulted in the formation of three DNA adducts.
enzymes can activate a variety of estrogenic compounds to form DNA adducts (28–31). These findings suggest that uterine peroxidase may play an important role in the activation of TAM metabolites to form DNA adducts in vivo.

Treatment of female rats with varying doses of TAM for 7 days produced a complex pattern of 11 DNA adducts and a dose dependent increase in DNA adduct levels in the liver. This observation is similar to what has been reported in other studies (9,12–14). The complexity of the DNA adduct pattern observed both in vivo and following microsomal activation of TAM (33,34,36) suggests that TAM is converted to multiple metabolites which are subsequently activated to form DNA adducts. In our own studies, we have presented evidence that 4-HO-TAM leads to the formation of DNA adduct 1 and that cis/trans-metabolite E results in the production of adducts 4 and 9 in the liver of TAM treated rats (33,34). Studies by Randerath et al. have demonstrated that administration of 4-HO-TAM results in the formation of one of the adducts observed after administration of TAM (40). Recent investigations by Phillips et al. have suggested that α-hydroxy-TAM may lead to the formation of the principal DNA adduct in cultured hepatocytes (41,42).

In contrast to the results with the liver, administration of 5 and 10 mg/kg of TAM for 7 days to the animals did not produce detectable DNA adducts in uterus. However treatment with 20 mg/kg TAM produced one adduct in the uterus with a relative adduct level of $0.64 \times 10^{-8}$. Rechromatography experiments demonstrated that adduct 1 found in the uterus was the same as adduct a produced by uterine extract activation of 4-HO-TAM. The DNA adducts formed by cis/trans-metabolite E were not detected in the uterus under these treatment conditions. These results suggest that 4-HO-TAM is the main metabolite of TAM leading to the formation of DNA adducts in the rat uterus. Comparison of the levels of DNA adducts produced in the liver with those in the uterus shows that the levels of adducts in the liver are ~36-fold higher. Similar experiments by Gladek and Liehr demonstrated a 10-fold difference in adduct levels in the liver and uterus of hamsters following a single treatment with DES (43).

Our results are consistent with the high levels of TAM and its metabolites detected in rat liver following treatment with TAM (9,44,45) and the observations that the liver is the primary site for the toxic effects of TAM in the rat (8–10). DNA adducts were observed in the uterus only at 20 mg/kg dose. A previous study has reported finding no DNA adducts in the uterus of rats or mice following gavage administration of TAM (13). Differences in the route of administration and strain of rat may account for the difference in results between the present study and those previously reported. The observation that DNA adducts were detected only at the highest dose suggests that either high doses or long term administration of lower doses of TAM may be required for sufficient accumulation of 4-HO-TAM in the uterus to result in DNA adduct formation.

In Figure 5, we present a working model for the formation of DNA adducts in the uterus after administration of TAM. In this model, initial metabolism of TAM occurs in the liver with subsequent accumulation of 4-HO-TAM in the uterus. It is possible that metabolism of TAM in the uterus may also contribute to the accumulation of 4-HO-TAM (46,47). The P450 responsible for the formation of 4-HO-TAM remains to be defined (48,49). In cells containing peroxidase enzymes, hydroperoxides formed by either endogenous metabolism (50), or by treatment with carcinogens (51–53) are reduced by...
peroxidase enzymes (54). As a result of this process the peroxidase enzyme is converted to an intermediate termed Compound I (54). A variety of estrogenic compounds can serve as reducing agents for this intermediate and in the process be oxidized to either semiquinones or quinones (55–58). These semiquinones or quinone intermediates may react with DNA to form DNA adducts (28–31). Recently our laboratory (34) and Moorhy et al. (59) have presented evidence that the quinone methide derivative of both 4-HO-TAM and cis\trans-metabolite E is the reactive intermediate leading to DNA adduct formation.

Recent studies have demonstrated that long term administration of TAM is associated with up to a 6-fold increase in endometrial cancer (4–7). The results of this study suggest that the formation of DNA adducts in uterine tissues following treatment with TAM may contribute to the observed increased risk.

Acknowledgements

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References


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