Determination and Pharmacology of a New Hydroxylated Metabolite of Tamoxifen Observed in Patient Sera during Therapy for Advanced Breast Cancer

V. Craig Jordan, Richard R. Bain, Raymond R. Brown, Barbara Gooßen, and M. Amparo Santos

ABSTRACT

A new hydroxylated metabolite of tamoxifen, Metabolite Y [trans-1-(p-β-hydroxyethoxyphenyl)-1,2-diphenylbut-1-ene] was characterized and subsequently measured by high-performance liquid chromatography in serum from patients receiving normal (10 mg twice daily) and high dose (150 mg twice daily) tamoxifen therapy for treatment of advanced breast cancer. In normal-dose patients, the serum level of Metabolite Y ranged between 6 and 60 ng/ml. This contrasted with serum levels of 80 to 180 ng/ml for tamoxifen and 200 to 300 ng/ml for N-desmethyltamoxifen, the major metabolite of tamoxifen. Serum levels of all three compounds were unchanged in one patient during the 24 hr after the cessation of tamoxifen therapy. Maximum serum levels of Metabolite Y were 800 ng/ml with concentrations of 1 µg/ml for tamoxifen and 2 µg/ml for N-desmethyltamoxifen in a patient on a 2-year course of high-dose therapy. Metabolite Y inhibited the binding of 17β-[3H]-estradiol to rat uterine and human breast carcinoma estrogen receptor. However, this metabolite was only weakly active: monohydroxytamoxifen [relative binding affinity (RBA) = 280]; tamoxifen (RBA = 6); Metabolite E (RBA = 3); N-desmethyltamoxifen (RBA = 4); Metabolite Y (RBA = 0.5). In 3-day immature rat uterine weight tests, Metabolite Y was a partial agonist with weak antiestrogenic activity. Although Metabolite Y has only weak activity, this compound would be expected to contribute to the overall antiestrogenic and antitumor properties of tamoxifen during therapy.

INTRODUCTION

Tamoxifen, a nonsteroidal antiestrogen, is used in the palliative treatment of advanced breast cancer (18) and as an adjuvant therapy after mastectomy (11). Although tamoxifen (Nolvadex) has been used clinically for more than a decade, there has been only recent interest in the pharmacokinetics and metabolism of the drug in patients.

An early study by Fromson et al. (12) documented several metabolites of tamoxifen in different laboratory species (Chart 1). Monohydroxytamoxifen is a potent antiestrogen with an estrogen receptor binding affinity similar to that of estradiol (17). This finding stimulated interest in the role of metabolism in the antiestrogenic and antitumor properties of tamoxifen (3, 5, 7, 10). Originally, it was thought that monohydroxyltamoxifen was the major nonconjugated metabolite of tamoxifen in breast cancer patients (13). Recently, however, monohydroxytamoxifen has been shown to be a minor metabolite, and N-desmethyltamoxifen has been confirmed as the major metabolite of tamoxifen in patients undergoing therapy for breast cancer (1, 8, 10). Metabolite D and Metabolite E have been detected only in laboratory animals (Chart 1) (12).

In order to understand the metabolism and drug interaction of tamoxifen in combination with chemotherapeutic regimens, we have refined and improved assays to monitor serum levels of tamoxifen and its metabolites in patients (6). During the development and validation of the assays, we observed a new metabolite of tamoxifen in the serum of patients receiving high-dose tamoxifen therapy (150 mg twice daily) for breast cancer. This new metabolite has been designated Metabolite Y (Chart 1). The structure of this metabolite has been confirmed (4) by comparison with synthetic standards using HPLC and gas chromatography-mass spectrometry.

We now describe the serum levels of tamoxifen and its metabolites, N-desmethyltamoxifen and Metabolite Y, in patients receiving normal (10 mg twice a day) and high-dose (150 mg twice a day) tamoxifen therapy. The estrogenic and antiestrogenic properties of Metabolite Y are compared with tamoxifen, monohydroxytamoxifen, N-desmethyltamoxifen, and Metabolite E to evaluate its pharmacology and its potential contribution to the actions of the parent drug.

MATERIALS AND METHODS

All solvents were HPLC grade from Burdick and Jackson Laboratories, Muskegon, Mich. Diethylamine and triethylamine were from Aldrich Chemicals, Milwaukee, Wis. 17β-Estradiol and chemicals for buffer solutions were from Sigma Chemical Co., St. Louis, Mo. Tamoxifen, N-desmethyltamoxifen, ICI 99,311 [1-(p-β-dimethylaminooethoxyphenyl)-1,2-diphenylacrylonitrile], Metabolite E, Metabolite Y, and monohydroxytamoxifen were gifts from ICI, Ltd. (Pharmaceuticals Division), Macclesfield, Cheshire, England.

Chromatographic Analysis. The TLC assay is an adaptation of that described by Adam et al. (2). Serum (0.1 to 0.5 ml) was spiked with 50 ng ICI 99,311 and extracted with 2 ml hexane:butanol (98:2) by continuous agitation for 30 sec on a Vortex mixer. Sealed tubes were centrifuged at 1000 x g for 10 min, and the organic phase was then evaporated to dryness under N2 at 55°C. The dried extract was redisolved in 50 µl absolute methanol and mechanically spotted (TLC Multispotter; Analytical Instrument Systems, Chicago, Ill.) onto silica
Inhibition of 17\(\beta\)\([\text{H}]\)estradiol Binding in Vitro. Frozen immature rat uterus (−70°C) were homogenized with a Polytron tissue homogenizer (2 × 10−6 sec bursts at 0 to 2°C). A ratio of 2 uteri per ml of TED buffer was used for all experiments. The homogenate was immediately centrifuged at 2000 × g (4°C) for 10 min. The supernatants were then decanted into scintillation vials and counted using 10 ml ACS (American) centrifuged at 100,000 × g for 1 hr (4°C) in a Sorvall OTD65 ultracentrifuge. The homogenate was immediately centrifuged at 2000 × g (4°C) for 10 min. The supernatants were then decanted into scintillation vials and counted using 10 ml ACS (American). The RBA for each competing ligand was calculated as follows:

\[
\text{RBA} = \frac{\text{Molar concentration of competitor for 50% inhibition of specific binding}}{\text{Molar concentration of } 17\beta\text{-estradiol for 50% inhibition of specific binding}} \times 100
\]

\[
\text{% Inhibition of } 17\beta\text{-}[\text{H}]\text{estradiol} = \frac{\text{Specific dpm for competitor tubes}}{\text{Specific dpm for control tubes}} \times 100
\]

RESULTS

Determination of Metabolites in Patient Serum. TLC was initially used to determine the levels of tamoxifen and N-desmethyltamoxifen in patients receiving tamoxifen 10 mg twice a day during a 1-year period. These results have been reported elsewhere (16) and confirm the earlier findings from other laboratories (2, 8, 9). In one patient (L. F.), the daily dose of tamoxifen was escalated in an attempt to control metastatic lesions in the brain. No tamoxifen was detected in cerebrospinal fluid; however, serum levels of tamoxifen and N-desmethyltamoxifen increased dramatically (Chart 2). The ability to detect monohydroxytamoxifen using TLC in normal-dose patients was variable, but low concentrations were detected upon dose escalation. In contrast, substantial quantities of an unidentified metabolite were observed, which increased with dose escalation of tamoxifen (Chart 3). As described previously (4), the unidentified metabolite had the same R\(_t\) as both Metabolite E and Metabolite Y using TLC. However, Metabolite E and Metabolite Y can be distinguished in this system by their changes in fluorescence intensities in acid conditions. Metabolite E is virtually undetectable under basic conditions conferred by residual triethylamine on the silica matrix. Coating the plate with hexane:paraffin oil:acetic acid (80:20:0.5) causes a 10-fold increase in fluorescence intensity for Metabolite E alone. Metabolite Y is insensitive to changes in pH. The detection of the unidentified metabolite was essentially unchanged under acidic or basic conditions. The identification of Metabolite Y was confirmed by HPLC purification and gas chromatography-mass spectrometry in Patient I. J. (4). HPLC was used to monitor the serum levels of tamoxifen, N-desmethyltamoxifen, and Metabolite Y in high- and normal-dose patients. During the treatment of a breast cancer patient with tamoxifen (170 mg twice a day), the serum levels of N-desmethyltamoxifen were observed to be approximately twice the serum levels achieved with the parent drug (Chart 4). As in
the original protocol because of indications that 20 mg twice a day did not control the disease. After treatment failure 5 months later, tamoxifen therapy was stopped, and blood samples continued to be drawn to determine tamoxifen and metabolites. While tamoxifen was virtually undetectable 30 days after therapy was stopped, substantial quantities of N-desmethyltamoxifen (1016 ng/ml) and Metabolite Y (350 ng/ml) were observed.

The levels of N-desmethyltamoxifen were approximately twice those observed for tamoxifen during 9 months of therapy with tamoxifen (10 mg twice a day) (Table 1). As noted previously (16), the serum levels were remarkably stable throughout this period. The serum levels of Metabolite Y varied considerably from patient to patient and ranged from 6 to 50 ng/ml (Table 1). In another patient, therapy with tamoxifen (10 mg twice a day) was stopped after 6 weeks, and the serum levels of tamoxifen, N-desmethyltamoxifen, and Metabolite Y were

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**Table 1**

<table>
<thead>
<tr>
<th>Serum levels (ng/ml)</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days 77</td>
<td>80</td>
<td>204</td>
<td>5</td>
</tr>
<tr>
<td>Days 119</td>
<td>189</td>
<td>265</td>
<td>5</td>
</tr>
<tr>
<td>Days 245</td>
<td>165</td>
<td>226</td>
<td>6</td>
</tr>
<tr>
<td>Days 278</td>
<td>158</td>
<td>242</td>
<td>6</td>
</tr>
<tr>
<td>Days 34</td>
<td>77</td>
<td>163</td>
<td>35</td>
</tr>
<tr>
<td>Days 90</td>
<td>87</td>
<td>229</td>
<td>49</td>
</tr>
<tr>
<td>Days 195</td>
<td>81</td>
<td>202</td>
<td>32</td>
</tr>
<tr>
<td>Days 279</td>
<td>78</td>
<td>179</td>
<td>12</td>
</tr>
<tr>
<td>Days 35</td>
<td>123</td>
<td>208</td>
<td>15</td>
</tr>
</tbody>
</table>

**ND**: not done.

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**Chart 2.** Serum levels of tamoxifen, N-desmethyltamoxifen, and monohydroxytamoxifen in Patient L. F. after 24 weeks of 10 mg twice daily tamoxifen but during 2 months of dosage escalation first to 30 and then 150 mg tamoxifen twice daily. Tamoxifen and metabolites were extracted from 200 μl serum with 2 ml diethyl ether using ICI 99311 as an extraction standard. The extract was dried down under N2 and redissolved in 50 μl absolute methanol. The extracts were spotted onto silica TLC plates and developed using a toluene:triethylamine: ethanol solvent system (90:10:1). After solvent evaporation, 20% paraffin oil in hexane was applied to plates and irradiated with UV light to convert the triphenylethylenes to phenanthrenes. Tamoxifen and metabolites were determined by fluorescence scanning of the plates and quantitated by comparison with standard curves for each compound.

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**Chart 3.** Representative fluorescence extracts of TLC plates to illustrate the increase in an unidentified fluorescent peak observed in the serum of Patient L. F. during the dosage escalation of tamoxifen. Serum was extracted and the plates were developed as described in Chart 2.

Patient L. F., the levels of Metabolite Y were substantial (between 600 and 800 ng/ml). Reduction of the daily dose of tamoxifen to 20 mg twice a day caused a consistent fall in the levels of tamoxifen, N-desmethyltamoxifen, and Metabolite Y. The dose of tamoxifen was reduced to avoid possible toxic side effects from long-term high-dose therapy on this therapeutic protocol. The patient was carefully monitored, but no adverse drug-related effects were observed. However, the dosage of tamoxifen was again increased to 170 mg twice a day as per
monitored over the next 24 hr. The serum levels of all 3 components were very stable with only a slight decline in tamoxifen (in the range 95 to 120 ng/ml) and N-desmethyltamoxifen (in the range 150 to 250 ng/ml) during the observation period. Metabolite Y levels remained constant during the 24 hr at around 6 ng/ml serum.

**Biological Properties of Tamoxifen and Metabolites.** The ability of tamoxifen and its metabolites to inhibit the binding of 17β-[3H]estradiol to estrogen receptors from rat uteri and MCF7 breast cancer cells was determined. The results, using rat or human estrogen receptor preparations, were the same, and only the comparison using MCF7 cytosol is illustrated in Chart 5. Monohydroxytamoxifen (RBA = 280) was more potent as a competitive ligand than 17β-estradiol (RBA = 100). Tamoxifen (RBA = 6), N-desmethyltamoxifen (RBA = 4), Metabolite E (RBA = 3), and Metabolite Y (RBA = 0.5) were low-affinity competitive ligands. The RBAs were consistent with the relative potencies of the compounds as antiestrogens in the 3-day immature rat uterine weight test (Chart 6). Monohydroxytamoxifen was the most potent antiestrogen, and Metabolite Y was the least potent. There was little difference between the antiestrogenic actions of tamoxifen and N-desmethyltamoxifen. However, the RBA values of the metabolites cannot be used to predict the biological activity of the metabolites. Monohydroxytamoxifen, tamoxifen, N-desmethyltamoxifen, and Metabolite Y were all partial estrogen agonists whereas Metabolite E was fully estrogenic compared with estradiol (Chart 7) in the rat uterine weight test.

**DISCUSSION**

The identification of Metabolite Y in patient serum during the therapy of breast cancer with tamoxifen (4) necessitated the comparative evaluation of its pharmacology with the other known metabolites of tamoxifen. The aim was to determine the estrogenic and antiestrogenic activity of the new metabolite, so its contribution to the pharmacology of tamoxifen in vivo could be assessed. Clearly, this could not be achieved without an estimate of the relative circulating concentration of the parent compound and its metabolites. Compared with N-desmethyltamoxifen, the major metabolite of tamoxifen in patients, Metabolite Y is a minor metabolite during normal-dose therapy. Nevertheless, considerable serum levels of Metabolite Y were achieved if the daily dose of tamoxifen was escalated. The pharmacology of tamoxifen and N-desmethyltamoxifen is very similar (Charts 5 to 7), and it is interesting to note that the deaminated product (Metabolite Y) retains antiestrogenic activity. The present result is supported by the recent findings of...
Robertson et al. (19) who have tested the synthetic compound during a study of the relationship of the basicity of the tamoxifen side chain and antiestrogenic activity.

The weak antiestrogenic activity of Metabolite Y in the uterine weight test may in fact result from the rapid excretion of this primary alcohol. Indeed, the data on the levels of metabolite Y in patients may not reflect the situation within target tissue cells. Studies on the direct effects of the metabolites of tamoxifen on the growth of MCF7 cells in culture indicate that tamoxifen and Metabolite Y have a similar potency.

Although a large number of structural modifications of tamoxifen’s side chain are possible without the loss of antiestrogenic activity (19) it is clear that complete removal of the alkylaminoethoxy side chain to form the phenol, Metabolite E, is disadvantageous for antiestrogenic activity. Metabolite E was estrogenic in the rat uterine weight test (Chart 7) and is also an estrogen in vitro. This observation is consistent with the literature on the estrogenic triphenylethylenes (for review, see Ref. 15). It should be pointed out the Metabolite E has not been identified as a metabolite of tamoxifen in patients. However, Metabolite E is present in dog bile (12). Since tamoxifen is an estrogen in the dog (14) it is interesting to speculate that the species differences in the pharmacology of the drug (15) may be the result of species differences in the metabolism of tamoxifen.

Although a novel hydroxylated metabolite of tamoxifen with antiestrogenic properties has been identified and quantitated in patients undergoing therapy for breast cancer, there are several unresolved issues that require further investigation. Clearly, the metabolic route for the conversion of tamoxifen to Metabolite Y requires description. Furthermore, since Metabolite Y appears to be less active than tamoxifen, it would be valuable to know whether any particular drug combinations resulted in the complete conversion of tamoxifen to a less active agent.

ACKNOWLEDGMENTS

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