Formation of Tamoxifen-DNA Adducts in Multiple Organs of Adult Female Cynomolgus Monkeys Dosed with Tamoxifen for 30 Days


ABSTRACT

The use of the antiestrogen tamoxifen (TAM) is associated with an increase in endometrial cancer. TAM-induced endometrial carcinogenesis may proceed through a genotoxin-mediated pathway, although the detection of endometrial TAM-DNA adducts in exposed women is still controversial. In this study, a monkey model has been used to investigate the question of TAM-DNA adduct formation in primates. Two methods have been used to determine TAM-DNA adducts: a TAM-DNA chemiluminescence immunoassay (TAM-DNA CIA), using an antiserum that has specificity for (E)-α-(deoxyguanosin-ι-N²)-tamoxifen (dG-TAM) and (E)-α-(deoxyguanosin-ι-N²)-N-desmethy1tamoxifen (dG-desmethyl-TAM) and electrospary ionization tandem mass spectrometry (ES-MS/MS) coupled with on-line sample preparation and high-performance liquid chromatography (HPLC). Mature (19 year old) cynomolgus monkeys were given either vehicle control (n = 1) or TAM (n = 3) twice daily for a total dose of 2 mg of TAM/kg body weight (bw)/day for 30 days by naso-gastric intubation. Tissues were harvested, and DNA was isolated from uterus, ovary, liver, brain cortex, and kidney. By TAM-DNA CIA, values for uterine TAM-DNA adducts in two monkeys were 0.9 and 1.7 adducts/10⁸ nucleotides. Liver DNA contained the highest TAM-DNA adduct levels (7.0–11.1 adducts/10⁸ nucleotides), whereas values for ovarian TAM-DNA adducts in the same animals were 0.4 and 0.5 adducts/10⁸ nucleotides. Liver, brain cortex, and kidney DNA samples from the three exposed monkeys had TAM-DNA levels of 2.1–4.2 adducts/10⁸ nucleotides, 0.4–5.0 adducts/10⁸ nucleotides, and 0.7–2.1 adducts/10⁸ nucleotides, respectively. By HPLC-ES-MS/MS, the levels of TAM-DNA adducts detected in all tissues were comparable with those observed by TAM-DNA CIA. Thus, values for uterine TAM-DNA adducts ranged from 0.5 to 1.4 adducts/10⁸ nucleotides, whereas values for ovarian TAM-DNA adducts, measurable in two monkeys, were 0.2 and 0.3 adducts/10⁸ nucleotides. Liver DNA contained the highest TAM-DNA adduct levels (7.0–11.1 adducts/10⁸ nucleotides), whereas brain cortex DNA contained lower adduct levels (0.6–4.8 adducts/10⁸ nucleotides) and the lowest levels were measured in the kidney (0.2–0.4 adducts/10⁸ nucleotides). This study indicates that cynomolgus monkeys are capable of metabolizing TAM to genotoxic intermediates that form TAM-DNA adducts in multiple tissues.

INTRODUCTION

One of every eight American women will develop breast cancer by approximately age 70, and 1,000,000 new breast cancer cases are diagnosed each year worldwide. The American Society of Clinical Oncology recently estimated that 700,000 women with breast cancer in the United States are currently receiving TAM, a synthetic antiestrogen containing mixed estrogen agonist and antagonist activity. TAM, an effective therapy for all stages of breast cancer, received Food and Drug Administration approval for adjuvant treatment in 1977 and for chemoprevention in healthy “at-risk” women in 1998. The current recommendation for treatment of estrogen receptor positive pre- and post-menopausal women is 20 mg of TAM/day for 5 years. At this dose, TAM was found to reduce the incidence of contralateral breast cancer by 47% (1) and to reduce breast cancer incidence in “at-risk” women by 49% (2).

It has been well established that TAM is a potent rat hepatocarcinogen. Several laboratories have demonstrated that TAM acts as a classical genotoxic chemical carcinogen in rat liver by exerting its carcinogenic activity through TAM-DNA adduct formation (3–7). Rats exposed to TAM have dose-related increases in hepatic TAM-DNA adducts (8) and liver tumors (3), and there is an association between hepatic TAM-DNA adduct levels and liver tumor incidence (9). In contrast, hepatic TAM-DNA adduct levels are low in TAM-exposed mice (8, 10), and no mouse liver carcinogenicity has been associated with TAM exposure (11). Neonatal TAM exposures cause reproductive organ tumors in both adult rats (9, 12) and adult mice (13).

In humans, epidemiological evidence links TAM exposure to increases in endometrial and, rarely, uterine cancer in breast cancer patients. The relative risk of endometrial cancer for TAM users is estimated to be 1.3–7.5 (2, 14, 15), and the prophylactic use of TAM is associated with a 2.5-fold increase in endometrial tumors (2). Current estimates are that of 1000 women receiving TAM, two to three will develop endometrial cancer (16). Furthermore, recent studies have revealed an association with an increase in uterine sarcoma (17), although this is reported to impact only 0.01% of the women receiving TAM (18).

There are two possible mechanisms of endometrial/uterine tumor induction: (a) an estrogenic pathway and (b) a pathway involving genotoxic intermediates that damage DNA and induce mutations. As an estrogen agonist in the uterus, TAM can promote cellular proliferation. As a genotoxin, TAM is metabolized to reactive intermediates capable of binding to DNA. DNA adduct formation constitutes an initial step in the carcinogenic process, as replication of a damaged DNA template can lead to the incorporation of an incorrect base, mutagenesis in critical genes, and a heritable loss of growth control. Several laboratories have investigated TAM-DNA adduct formation in human endometrium and arrived at conflicting conclusions (5, 19, 20–24). To examine this issue in a primate model, three elderly (19 year old) cynomolgus monkeys were dosed by gavage for 30 days with 2 mg of TAM/kg bw/day, a daily dose ~6-fold greater than the human daily dose. We quantified dG-TAM and dG-desmethyl-TAM, the major DNA adducts detected in the livers of rats administered TAM, by TAM-DNA CIA (25) and by HPLC-ES-MS/MS (26). We hypothesized that if TAM-DNA adducts were measurable in tissues from TAM-exposed monkeys, primates, including humans, may have the capability to metabolize TAM into genotoxic intermediates.
MATERIALS AND METHODS

Chemicals. TAM and calf thymus DNA were from Sigma (St. Louis, MO). Opaque 96-well high binding plates were purchased from Greiner Labotechnik (PGC Scientific, Frederick, MD). Biotinylated antirabbit IgG, streptavidin-alkaline phosphatase, 1-Block (Casein) and CDP-Star with Emerald II were from Tropix (Bedford, MA). Amyloglucosidase was purchased from Boehringer Mannheim (Indianapolis, IN). Reacti-Bind DNA coating solution was obtained from Pierce (Rockford, IL). CIA wash buffer was obtained from KD Medical (Columbia, MD). PBS was from Life Technologies, Inc. (Grand Island, NY).

Animals. Four adult (19 year old) retired breeder female cynomolgus (Macaca fascicularis) monkeys were housed and treated at Conring Hazelton Laboratories (Vienna, VA). Animal care was provided in accordance with the standards established by the Association for Assessment and Accreditation for Laboratory Animal Care. The experimental protocols were approved by the Hazelton Animal Care and Use Committee. TAM (1 mg/kg bw) was administered to three monkeys twice daily (8 h apart) on weekdays and once on Saturday and Sunday by naso-gastric intubation as a suspension in 0.5% methyl cellulose at 2 ml/kg bw/dose. The total dose administered during the 30-day treatment was 52 mg of TAM/kg bw. The animals were euthanized 16–18 h after the last dose, and organs (brain cortex, liver, kidney, ovary, and uterus) were harvested for DNA isolation. DNA was isolated by nonorganic extraction (DNA Extraction Kit; Stratagene, La Jolla, CA), followed by digestion with 1 unit/ml amyloglucosidase (30 min at 37°C) in the case of DNA isolated from liver tissue. DNA was quantified by UV spectrophotometry at 260 nm.

TAM-DNA CIA. Rabbit antiserum elicited against DNA modified to 2.4% with dG-TAM, as determined by 32P-postlabeling, was used in the TAM-DNA CIA as described previously (25). In brief, DNA samples for coating plates were sonicated for 20 s and heat denatured for 5 min at 95°C. Each well of a 96-well microtiter plate was coated with 8.2 pg TAM-DNA in Reacti-Bind DNA coating solution. The coating was for 24–48 h at room temperature, and the plates were stored at −20°C until use. Nonspecific binding was reduced by incubating with 300 µl of casein solution/well (0.33% I-Block in PBS, 0.05% Tween 20, and 0.1% NaN3) for 90 min at 37°C. After each incubation, the plates were washed three times with CIA wash buffer [137 mM PBS, 1.5 mM KH2PO4, 8.1 mM Na2HPO4, 3 mM KCl, 0.02% NaN3, and 0.05% Tween 20 (pH 7.4)]. A TAM-DNA standard curve, which was included on each plate used for TAM-DNA CIA, was generated from a TAM-DNA standard, modified to 2.4% with dG-TAM, and diluted to 0.3 amol TAM-DNA/µl. The samples, each assayed on three wells coated with TAM-DNA and one well coated with non-TAM-DNA, were added to each well of the microtiter plate and incubated for 90 min at room temperature. After washing, streptavidin-alkaline phosphatase (100 µl, 1:3750 dilution in casein solution) was added and incubated for 60 min at room temperature. After a final wash, 100 µl of CDP-Star containing Emerald II enhancement solution was added. The plates were kept for 20 min at room temperature and overnight at 4°C. The next day, the plates were warmed to room temperature for 20 min, and the light emission was measured at 542 nm using a TR717 Microplate Luminometer (PE Applied Biosystems, Foster City, CA).

Sample quantitation was achieved by comparison of unknown samples with a TAM-DNA standard curve, in which 50% inhibition was at 0.86 ± 0.2 fmol dG-TAM (mean ± SE, n = 5). When 20 µg DNA was analyzed, the LOD was calculated to be 10 amol of dG-TAM adduct/µg DNA or 0.3 adducts/108 nucleotides.

HPLC-ES-MS/MS Analyses. The DNA adduct standards, dG-TAM and dG-desmethyl-TAM, were synthesized and characterized, and the DNA samples were hydrolyzed to nucleosides and analyzed as described (26). For each analysis, 80 µg DNA were hydrolyzed. All hepatic DNA samples were analyzed twice, and the rest of the DNA samples were analyzed once. In brief, each sample was loaded onto a reversed phase trap column [Luna C18 (2), 2 × 30 mm, 3 µm; Phenomenex, Torrance, CA] and eluted with 75% of 0.1% formic acid and 27% of acetonitrile at 0.2 ml/min into the mass spectrometer. A Quattro Ultima triple quadruple mass spectrometer (Micromass, Manchester, United Kingdom), equipped with an electrospray interface, was used with a source block of 150°C and a desolvation temperature of 450°C. Nitrogen was used as the desolvation (750 liters/h) and nebulizing (90 liters/h) gas. Argon was used as the collision gas, at a collision cell pressure of 1.5 × 10−3 mBar. Positive ions were acquired in the MRM mode (dwell time of 0.3 s, span of 0.02 Da, and interchannel delay of 0.03 s) for the (M + 2H)+ → (BH + 2H)+ transitions of dG-TAM (m/z 319 → 261), dG-desmethyl-TAM (m/z 312 → 254), and the internal standard, dG-TAM-d4 (m/z 322 → 264). The cone voltage was 15 V, and the collision energy was 9 eV for all three transitions.

RESULTS

Monkey Colony and TAM Exposure. The female cynomolgus monkeys used in this study were breeders that had participated previously in a terminated National Cancer Institute study. At 19 years of age, they were considered beyond midlife because the average life span is ~30 years. They were therefore assumed to provide an appropriate model for TAM use in breast cancer survivors. At the time these experiments were initiated, women were receiving 20 or 40 mg of TAM/day given in two doses (0.33–0.66 mg TAM/kg bw/day). We chose a daily TAM dose (2 mg/kg bw) that was ~6-fold higher than the human daily dose. In this study, monkeys (n = 3) were dosed with a total cumulative TAM dose of 52 mg/kg bw. One unexposed monkey received vehicle by naso-gastric intubation. DNA was prepared from brain cortex, liver, uterus, ovary, and kidney.

Detection of Adducts by TAM-DNA CIA. For each assay, microtiter plates contained a TAM-DNA standard curve as well as test samples, each assayed on three wells coated with TAM-DNA and one well coated with unmodified DNA. In addition, samples were assayed on two to three different microtiter plates on different days. The TAM-DNA antibody is specific for both dG-TAM and dG-desmethyl-TAM, presumably recognizing both adducts in biological samples, and the immunoassay LOD was 0.3 adducts in 108 nucleotides (25).

Values for TAM-DNA adducts determined using the TAM-DNA CIA are shown in Fig. 1A and listed in Table 1. The highest adduct levels were detected in the liver and brain cortex; all three animals gave measurable TAM-DNA levels in liver and brain cortex. Lower, but measurable, TAM-DNA levels were observed in the kidneys of all three exposed monkeys. The ovaries and uteri of two of the three animals had low but measurable levels of TAM-DNA adducts.

Determination of TAM-DNA Adducts by HPLC-ES-MS/MS. Monkey DNA samples were also analyzed using a newly developed assay that involves on-line HPLC separation and ES-MS/MS detection of TAM-DNA adducts, using a deuterated adduct as internal standard (26). This method recognizes dG-TAM and dG-desmethyl-TAM, presumably recognizing both adducts in biological samples, and the immunoassay LOD was 0.3 adducts in 108 nucleotides (25). Values for TAM-DNA adducts determined using the HPLC-ES-MS/MS method were remarkably similar to those observed with the TAM-DNA CIA. The data are shown in Fig. 1B and Table 1, and representative chromatograms are shown in Figs. 2 and 3. Again, the values in liver and brain cortex were the highest. There was an apparent discrepancy between the two methods for the values in the kidneys, because the HPLC-ES-MS/MS method gave values that were several-fold lower than those observed by TAM-DNA CIA in two of the three monkeys (Table 1). In the three exposed monkeys, DNA isolated from the uteri contained detectable dG-TAM levels by HPLC-ES-MS/MS, with values between 0.5 and 1.4 adducts/108 nucleotides (Table 1). Lower, but measurable, dG-TAM values were found in the ovaries of two monkeys. dG-desmethyl-TAM was not detected in any sample by HPLC-ES-MS/MS.
With the first set of liver DNA samples assayed using this method, the unexposed monkey (monkey number 07) gave a positive value of 5.1 adducts/10^8 nucleotides in the liver DNA. To determine whether this was an artifact caused by the analytical procedure, we subsequently assayed liver DNA samples from two additional unexposed monkeys (09 and 11) and did not observe positive values with either of those samples. We did, however, also find a value of 0.5 adduct/10^8 nucleotides in the brain of unexposed monkey 07 by HPLC-ES-MS/MS, whereas the values for brain cortex DNA from monkeys 09 and 11 were below the detection limit.

**DISCUSSION**

In this study, we have demonstrated the formation of TAM-DNA adducts in a monkey model using two methodological approaches: TAM-DNA CIA and HPLC-ES-MS/MS. Monkeys were exposed to 2 mg of TAM/kg bw daily for 30 days. The dose used (2 mg of TAM/kg bw/day) was ~6-fold higher than the current recommended daily dose for women. However, the monkeys in this study were only exposed for 1 month, whereas breast cancer patients typically receive TAM for 5 years. TAM-DNA adducts were found in most tissues examined, with the highest levels being detected in the liver and brain cortex. Although TAM-DNA CIA detected adduct levels of 0.7–2.1/10^8 nucleotides in kidney DNA samples, lower levels (0.2–0.4 adducts/10^8 nucleotides) were measured by HPLC-ES-MS/MS. If the major kidney DNA adduct was dG-desmethyl-TAM, it would not be detected by HPLC-ES-MS/MS at the levels indicated by TAM-DNA CIA. Most importantly, TAM-DNA adducts were found in the uteri and ovaries of all three exposed animals. The data demonstrate that nonhuman primates are capable of metabolizing TAM into genotoxic intermediates that form DNA adducts in suspected target tissues. The data also suggest that humans may have the metabolic capacity to form TAM-DNA adducts in organs at risk for TAM-induced cancers.

In addition to the TAM-DNA CIA and HPLC-ES-MS/MS analyses reported here, DNA samples from this study have been assayed by 3^P-postlabeling-HPLC by Shibutani et al. (27). The method differentiates between dG-TAM and dG-desmethyl-TAM and determines both adducts with similar sensitivity. The combined values for the two adducts were very similar to those reported here using TAM-DNA CIA and HPLC-ES-MS/MS. In the liver, all three exposed monkeys

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**Table 1** Comparison of monkey TAM-DNA adducts (per 10^8 nucleotides) by two different methods

<table>
<thead>
<tr>
<th>Organ</th>
<th>Monkey number</th>
<th>TAM exposure</th>
<th>TAM-DNA CIA</th>
<th>HPLC-ES-MS/MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uterus</td>
<td>07</td>
<td>−</td>
<td>ND^a</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>+</td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>04</td>
<td>+</td>
<td>ND</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>+</td>
<td>1.7</td>
<td>1.4</td>
</tr>
<tr>
<td>Liver</td>
<td>07</td>
<td>−</td>
<td>ND</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>09</td>
<td>−</td>
<td>NA^a</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>−</td>
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<td>ND</td>
</tr>
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<td></td>
<td>14</td>
<td>+</td>
<td>3.7</td>
<td>10.6</td>
</tr>
<tr>
<td></td>
<td>04</td>
<td>+</td>
<td>2.1</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>+</td>
<td>4.2</td>
<td>11.1</td>
</tr>
<tr>
<td>Brain cortex</td>
<td>07</td>
<td>−</td>
<td>ND</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>+</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>04</td>
<td>+</td>
<td>5.0</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>+</td>
<td>2.3</td>
<td>2.2</td>
</tr>
<tr>
<td>Ovary</td>
<td>07</td>
<td>−</td>
<td>NS^b</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>+</td>
<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>04</td>
<td>+</td>
<td>ND</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>+</td>
<td>0.5</td>
<td>ND</td>
</tr>
<tr>
<td>Kidney</td>
<td>07</td>
<td>−</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>+</td>
<td>0.7</td>
<td>0.3</td>
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<tr>
<td></td>
<td>04</td>
<td>+</td>
<td>2.1</td>
<td>0.4</td>
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<td></td>
<td>15</td>
<td>+</td>
<td>1.3</td>
<td>0.2</td>
</tr>
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</table>

^a ND, not detectable.
^b NA, not assayed.
^c NS, no sample.
were positive, with a value of 4.43 ± 0.37 adducts/10^8 nucleotides (mean ± SD), whereas the brain cortex had 1.45 ± 0.91 adducts/10^8 nucleotides. One monkey had measurable adducts in the uterus (0.52 adducts/10^8 nucleotides), and two monkeys had measurable adducts in the ovaries (0.20 and 0.42 adducts/10^8 nucleotides). There were no adducts detected in the kidneys. For 32P-postlabeling-HPLC, the LOD varied with the tissue examined and was between 0.1 and 0.4 adducts/10^8 nucleotides. Because all three methods for determination of TAM-DNA adducts gave consistent positive values for monkey liver, brain cortex, uterus, and ovary, it is clear that the cynomolgus monkey is capable of metabolizing TAM into intermediates that form DNA adducts in many different organs, including organs at risk for cancer induction in humans.

Rats exposed to TAM develop liver (3, 28) and uterine tumors (12) and have high levels of hepatic TAM-DNA adducts (4, 8, 29, 30). However, some laboratories have found no evidence of rat uterine TAM-DNA adducts (31, 32), whereas other groups have reported TAM-DNA adduct formation in this tissue (33, 34). Unlike rats, TAM-exposed mice are not susceptible to liver tumor induction (11) and have low levels of TAM-induced hepatic DNA adducts (8, 10). Similar to rats, the exposed mice develop uterine tumors, but there has been no evidence of uterine TAM-DNA adducts (35). One study examined TAM-DNA adduct formation in rhesus monkeys (36), but because this was a short-term exposure, it was not possible to ascertain tumor formation in either the liver or uterus. Rhesus monkeys were given two daily doses of 25 mg/kg bw for 1 week, and exposed male and female monkeys had hepatic TAM-DNA adduct levels of 10 and 14/10^8 nucleotides, respectively, when assessed by 32P-postlabeling. In this study, the unexposed controls had background levels of ~3.5 adducts/10^8 nucleotides (36). In addition, the daily TAM dose chosen was 10-fold higher than the dose used for the experiments reported here.

There are strong associations between DNA damage, mutagenesis, and carcinogenesis. These associations are clearly evident in livers of rats exposed to TAM; however, in humans, there is controversy surrounding the detection of endometrial TAM-DNA adducts. Several groups have reported the formation of TAM-DNA adducts in leukocytes (37, 38) and human endometrial tissue (19–21). However, other groups have not detected TAM-specific human DNA adducts in either endometrium or leukocytes (5, 22, 23, 24, 39, 40). Humans exposed to TAM do not demonstrate an increased incidence of liver cancer (1), and no TAM-specific DNA adducts have been detected in liver tissue from biopsies of several patients on TAM therapy (41). In one study, α-hydroxy-TAM was present in human endometrial explant cultures, although no DNA adducts were detected in explants exposed to 20–500 µM TAM (22). Therefore, although the formation of TAM-DNA adducts in human endometrial tissue may be possible, sufficiently sensitive methods for their reliable detection may not be available.

Methods used for human TAM-DNA adduct quantitation have primarily utilized 32P-postlabeling. In 1996, Hemminki et al. (19) found positive TAM-DNA adducts in five of seven human endometrium samples at an adduct level of 2.7 in 10^7 nucleotides. However, the TAM-specific radioactivity was only twice the background, and its significance has been questioned (5, 42). Carmichael et al. (23, 43) did not find TAM-DNA adducts in human endometrium samples, even when using the protocol of Hemminki et al. (19). Shibutani et al. (20) reported that 6 of 13 TAM-exposed patients had endometrial TAM-DNA levels of 1.5–13.1 adducts/10^8 nucleotides, determined by 32P-postlabeling-TLC. In a second study (21), these same 13 samples were reanalyzed with three additional samples by an improved 32P-postlabeling-HPLC method, and 0.2–18 TAM-DNA adducts/10^8 nucleotides were detected in 8 of 16 samples. However, the cis/trans ratios of the TAM-DNA adducts reported in these studies were different from those reported to form chemically in tissue culture and animal models (5). Martin (44) et al. used the highly sensitive accelerator mass spectrometry, with a single 14C-labeled TAM exposure in hysterecomy patients, and reported very low levels of uncharacterized adducts in the endometrium and myometrium. These controversial findings may be influenced by multiple factors, including variable 32P-postlabeling phosphorylation efficiencies, and the detection procedures used in different laboratories.

Overall, the data presented here suggest that a genotoxic mechanism could be partially involved in the etiology of endometrial cancer in breast cancer patients receiving TAM therapy. Although TAM is very effective in inhibiting breast cancer induction, the use of this drug in healthy “at-risk” women may require monitoring for endometrial abnormalities. Additional studies are needed to understand fully the link between TAM exposure, TAM-DNA adduct formation in endometrium, and endometrial cancer.

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