Background: Although the nonsteroidal antiestrogen tamoxifen is used as an adjuvant chemotherapeutic agent to treat hormone-dependent breast cancer and as a chemopreventive agent in women with elevated risk of breast cancer, it has also been reported to increase the risk of endometrial cancer. Reports of low levels of tamoxifen–DNA adducts in human endometrial tissue have suggested that tamoxifen induces endometrial cancer by a genotoxic mechanism. However, these findings have been controversial. We used electrospray ionization–tandem mass spectrometry (ES-MS/MS) and 32P-postlabeling analyses to investigate the presence of tamoxifen–DNA adducts in human endometrial tissue. Methods: Endometrial DNA from eight tamoxifen-treated women and eight untreated women was hydrolyzed to nucleosides and assayed for [(E)–α-(deoxyguanosin-\N\-yl)-tamoxifen (dG-Tam)] and [(E)–α-(deoxyguanosin-\N\-ylyl)-(\N\-desmethy1tamoxifen (dG-desMeTam), the two major tamoxifen–DNA adducts that have been reported to be present in humans and/or experimental animals treated with tamoxifen, using on-line sample preparation coupled with high-performance liquid chromatography (HPLC) and ES-MS/MS. The same DNA samples were assayed for the presence of dG-Tam and dG-desMeTam by 32P-postlabeling methodology, using two different DNA digestion and labeling protocols, followed by both thin-layer chromatography and HPLC. Results: We did not detect either tamoxifen–DNA adduct by HPLC–ES-MS/MS analyses (limits of detection for dG-Tam and dG-desMeTam were two adducts per 10^8 nucleotides and two adducts per 10^8 nucleotides, respectively) or by 32P-postlabeling analyses (limits of detection for both adducts was one adduct per 10^9 nucleotides) in any of the endometrial DNA samples. Conclusion: The initiation of endometrial cancer by tamoxifen is probably not due to a genotoxic mechanism involving the formation of dG-Tam or dG-desMeTam. [J Natl Cancer Inst 2004;96:1099–1104]
graphed before it was analyzed by accelerator mass spectrometry, making it unclear whether the [14C] that was measured represented covalently bound tamoxifen–DNA adducts or noncovalently bound metabolites of tamoxifen that were intercalated within the DNA.

With the exception of the study by Martin et al. (16), all analyses reported to date of human endometrial DNA from tamoxifen-treated patients have used a 32P-postlabeling method to detect tamoxifen–DNA adducts. Although this method is quite sensitive, it does not allow an unequivocal characterization of tamoxifen–DNA adducts. We recently developed an assay for quantifying tamoxifen–DNA adducts that couples on-line sample preparation with HPLC and electrospray ionization–tandem mass spectrometry [HPLC–ES–MS/MS; (24)]. By using a deuterated (E)-α-(deoxyguanosin-N2-yl)-tamoxifen (dG-Tam) adduct as an internal standard and multiple reaction monitoring, we have obtained high chemical specificity for dG-Tam and (E)-α-(deoxyguanosin-N2-yl)-N-desmethyltamoxifen (dG-desMeTam), the predominant DNA adducts detected in experimental animals treated with tamoxifen (25,26). In our initial studies, we used this methodology to assess the tamoxifen–DNA adduct levels in Sprague–Dawley rats (24) and cynomolgus monkeys (27) treated with tamoxifen. We have now used this technique to assay endometrial DNA from women who received tamoxifen therapy. For comparison, the same DNA samples were analyzed by 32P-postlabeling assays.

**MATERIALS AND METHODS**

**Chemicals**

Bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane (Bis–Tris), salmon testis DNA, proteinase K, RNase A, RNase T1, DNase I, snake venom phosphodiesterase I, and bacterial alkaline phosphatase were obtained from Sigma Chemical (St. Louis, MO). The following enzymes were used for 32P-postlabeling analyses: spleen phosphodiesterase (Calbiochem-Novabiochem, La Jolla, CA), or Worthington Biochemical, Lakewood, NJ), micrococcal nuclease (Roche Applied Science, Indianapolis, IN), nuclease P1 (Sigma), and T4 polynucleotide kinase (PNK; Epicentre Technologies, Madison, WI). Carrier-free [γ-32P]ATP was obtained from ICN Biomedicals (Irvine, CA).

**DNA Adduct Standards**

dG-Tam (24) and dG-desMeTam (26) were prepared as previously described. The deuterated adduct, (E)-α-(deoxyguanosin-N2-yl)-N,N-bis(3-deuteriethyl) tamoxifen (dG-Tam-d8), which was used as an internal standard in the HPLC–ES–MS/MS analyses, was synthesized from α-hydroxytamoxifen-d4 and isolated as previously described (24). For quantification purposes, we assumed that the molar extinction coefficients of dG-desMeTam and dG-Tam-d4 were identical to that previously determined for dG-Tam (24). Stock solutions containing known concentrations of dG-Tam, dG-desMeTam, or dG-Tam-d8 in methanol were prepared and stored at −20°C in vials sealed with teflon septa.

**Tissue Samples and DNA Isolation**

Written informed consent was obtained from each woman who provided a sample for this study. The study was reviewed and approved by institutional review boards at the National Center for Toxicological Research (U.S. Food and Drug Administration, Jefferson, AR); the Veterans Affairs Medical Center, Minneapolis, MN; the University of Minnesota, Minneapolis; and the Imperial College London, London, U.K.

Endometrial tissue was obtained from five women (mean age = 47 years, range = 37–57 years) who had taken tamoxifen (20 mg/day for 6–60 months) and were undergoing surgical procedures at the Fairview-University Medical Center, University of Minnesota. The tissues were provided by the Tissue Procurement Facility of the University of Minnesota Comprehensive Cancer Center. We also obtained endometrial tissue from five women (mean age = 42 years, range = 32–55 years) who had undergone hysterectomies for benign or malignant uterine or ovarian abnormalities at the same institution and had never taken tamoxifen. Nine of these tissue samples were histologically normal; the exception was a sample of malignant endometrium from a tamoxifen-treated woman. We also obtained endometrial tissue samples from three women (mean age = 65 years, range = 55–75 years) who had taken tamoxifen (20 mg/day for 15–37 months) and were undergoing surgical procedures at St. Helier Hospital, Carshalton, Surrey, U.K., and from three women (mean age = 48 years, range = 43–54 years) who had not taken the drug and had undergone hysterectomies at the same institution. The endometrial tissues from the U.K. were macroscopically normal.

We obtained two breast tissue samples that were taken from the contralateral breasts of two breast cancer patients who had undergone bilateral mastectomy at the Fairview-University Medical Center. One breast tissue sample came from a 51-year-old woman who had taken tamoxifen (20 mg/day) for 24 months, and the other came from a 40-year-old woman who had not taken tamoxifen. Both breast tissue samples were histologically normal. All tissue samples were stored at −70°C until DNA isolation, and all DNA samples were stored at −70°C until DNA adduct analysis.

The U.S. tissue samples (100–200 mg) were frozen in liquid nitrogen, pulverized with a Bessman tissue pulverizer (Fisher Scientific, Pittsburgh, PA), transferred to a glass homogenizer, homogenized in the presence of 1 mL of lysis buffer (500 mM Tris–HCl [pH 8.0], 20 mM EDTA, 10 mM NaCl, 1% sodium dodecyl sulfate), incubated with 1.4 mg of proteinase K at 37°C for 4 hours, and deproteinized by precipitation with saturated NaCl and centrifugation at 15 000g for 30 minutes at 4°C. The DNA was then precipitated with ethanol, collected by centrifugation at 15 000g for 15 minutes at 4°C, dissolved in 400 μL of 5 mM Bis–Tris and 0.1 mM EDTA (pH 7.1), incubated at 37°C for 2 hours with 270 μg of RNase A and 250 U of RNase T1, extracted with organic solvents (28), and precipitated with NaCl and ethanol. The DNA was collected by centrifugation at 20 000g for 10 minutes at 4°C, dissolved in 100–200 μL of 5 mM Bis–Tris and 0.1 mM EDTA (pH 7.1), and quantified by UV spectroscopy. DNA from the U.K. samples was isolated as described by Beland et al. (28).

**HPLC–ES–MS/MS Assay To Detect Tamoxifen–DNA Adducts**

DNA samples (40–100 μg) were hydrolyzed to deoxyribonucleosides (29) and analyzed by HPLC–ES–MS/MS as previously described (24). Briefly, each hydrolyzed DNA sample was
loaded onto a reverse-phase trap column [Luna C18(2), 2 mm × 30 mm, 3-µm particle size; Phenomenex, Torrance, CA]. After the trap column was washed, the flow was reversed and the sample was eluted through an analytical column [Luna C18(2), 2 mm × 150 mm, 3-µm particle size; Phenomenex] with a solution of 0.1% formic acid and acetonitrile (73%: 27%) at a flow rate of 200 µL/minute and into a Quattro Ultima triple quadrupole mass spectrometer (Micromass, Manchester, U.K.) equipped with an electrospray interface, with a source block of 120 °C and a desolvation temperature of 400 °C. Nitrogen was used as the desolvation (750 L/hour) and the nebulizing gas; argon was used as the collision gas (collision cell pressure = 1.5 µbar). Adducts were detected and identified by multiple reaction monitoring (dwell time = 0.3 seconds, span = 0.02 d, inter-channel delay = 0.03 seconds) and quantified by comparison to the internal standard dG-Tam-d6. Positive ions were acquired for the (M+2H)+→(BH+2H)+ transitions of dG-Tam (m/z 319→261), dG-desMeTam (m/z 312→254), and the internal standard, dG-Tam-d6 (m/z 322→264). The cone voltage was 15 V, and the collision energy was 9 eV for all three transitions. As positive controls, the following samples were analyzed concurrently: liver (≈10 dG-Tam per 108 nucleotides) and uterus (≈0.9 dG-Tam per 108 nucleotides) DNA from cynomolgus monkeys treated daily for 30 days with tamoxifen at 2 mg/kg of body weight (27), liver DNA (≈500 dG-Tam per 108 nucleotides and ≈625 dG-desMeTam per 108 nucleotides) from female Sprague–Dawley rats treated daily for 7 days with tamoxifen at 20 mg/kg of body weight (24), and salmon testis DNA (≈560 dG-Tam per 108 nucleotides) reacted in vitro with α-acetoxytamoxifen (24). The limit of detection, based on a signal-to-noise ratio of 3, was approximately two adducts per 109 nucleotides for dG-Tam and two adducts per 109 nucleotides for dG-desMeTam.

32P-Postlabeling Assay To Detect Tamoxifen–DNA Adducts

Each of the human endometrial and breast DNA samples was also assessed by 32P-postlabeling assays. In addition, a DNA sample from the liver of a male F344 rat that had received tamoxifen at 45 mg/kg of body weight daily by gavage for 14 days (30) was included as a positive control. We used two different protocols to digest and 32P-postlabel each DNA sample. Method A was based on a protocol resulting from an interlab trial on 32P-postlabeling methodologies (31). Briefly, each DNA sample (10 µg) was digested with 60 µL of spleen phosphodiesterase (Calbiochem) and 240 µL of micrococcal nuclease for 2 hours at 37 °C. The digest was further incubated with 443 µL of nuclease P1, 1 hour, then with 50 µCi of [γ-32P]ATP and 6 U of T4 PNK for 30 minutes at 37 °C, and the 32P-labeled digest was applied to a TLC plate. Chromatography was then conducted, and adducts were detected and quantified as described previously (32). Method B was based on procedures described by Shibutani et al. (14,33). Briefly, DNA (10 µg) was digested with 150 µL of spleen phosphodiesterase (Worthington) and up to 15 U of micrococcal nuclease for 2 hours at 37 °C, followed by incubation with 1 U of nuclease P1 for 1 hour at 37 °C. The digest was then extracted with butanol (14), the butanol was evaporated, and the residue was dissolved in water and incubated with 30 µCi of [γ-32P]ATP and 30 U of T4 PNK for 40 minutes at 37 °C. Each 32P-labeled digest was subjected to TLC by using 1.7 M sodium phosphate [pH 6.0] as the D1 solvent (14) and the D2 and D3 solvents described in (32).

The tamoxifen–DNA adducts were also analyzed by HPLC according to the procedure described in Shibutani et al. (14). Briefly, each 32P-labeled digest was applied to a TLC plate and eluted with 1.7 M sodium phosphate (pH 6.0). 32P-Labeled products were recovered from the origin, loaded onto a reverse-phase column (Jupiter C18, 4.6 mm × 250 mm, 5-µm particle size; Phenomenex), and eluted with a linear gradient of 10%–70% methanol in 200 mM ammonium formate (pH 4.0), over 30 minutes at a flow rate of 1 mL/minute. Adducts were detected and quantified with a flow scintillation analyzer (Canberra Packard, Pangbourne, U.K.). The limit of detection, based on a signal that was twice the background radioactivity, was approximately one adduct per 107 nucleotides for dG-Tam and dG-desMeTam for both the TLC and HPLC analyses.

RESULTS

We analyzed DNA isolated from endometrial tissue obtained from eight women who had taken therapeutic doses (20 mg/day) of tamoxifen for 6–60 months and from eight women who had not taken tamoxifen. We also analyzed DNA isolated from breast tissue obtained from one woman who had been treated with tamoxifen (20 mg/day) for 24 months and from one woman who had not been treated with tamoxifen.

HPLC–ES-MS/MS Analyses

Each of the DNA samples was enzymatically hydrolyzed to deoxynucleobases, mixed with the internal standard dG-Tam-d6, and subjected to HPLC–ES-MS/MS assays to detect dG-Tam and dG-desMeTam, the predominant tamoxifen–DNA adducts found in experimental animals and/or reported to be present in human endometrial DNA samples. Figure 1 shows representative multiple reaction monitoring chromatograms for control DNA from salmon testis (A) and for endometrial DNA from women who had (C) or had not (B) taken tamoxifen. The upper row of chromatograms presents the multiple reaction monitoring traces for the internal standard, dG-Tam-d6. The lower row of chromatograms presents the corresponding multiple reaction monitoring traces for dG-Tam. Figure 1, A, shows the multiple reaction monitoring traces obtained for 100 µg of salmon testis DNA plus 5 pg of dG-Tam and 25 pg of dG-Tam-d6. The amount of dG-Tam in this sample is equivalent to a DNA adduct level of 2.5 tamoxifen–DNA adducts per 108 nucleotides. We detected peaks corresponding to the internal standard dG-Tam-d6 (retention time = 8.18 minutes; upper row) and to dG-Tam (retention time = 8.19 minutes; lower row). Endometrial DNA samples from women who had (Fig. 1, C, top) or had not (Fig. 1, B, top) taken tamoxifen also had peaks corresponding to the internal standard dG-Tam-d6 (retention time = 8.14 minutes). We detected a peak in the 8.0- to 8.5-minute region of the m/z 319→261 multiple reaction monitoring traces of endometrial DNA from women who had taken tamoxifen (Fig. 1, C, bottom). This peak (retention time = 8.24–8.25 minutes), which consistently eluted after the peak corresponding to dG-Tam-d6, was also detected in endometrial DNA samples obtained from women who had not taken tamoxifen (Fig. 1, B, bottom). The same peak was also observed in the breast DNA sample from a woman who was treated with tamoxifen as well.
as in the breast DNA sample from a woman who did not receive tamoxifen (not shown). Because tamoxifen-treated and control samples gave essentially identical profiles, we conclude that neither dG-Tam (less than two adducts per \(10^9\) nucleotides) nor dG-desMeTam (less than two adducts per \(10^7\) nucleotides) was present in any of the endometrial DNA samples or in the single breast DNA sample from women who received tamoxifen therapy.

As positive controls, we concurrently analyzed DNA samples isolated from monkey livers and uteri and from the livers of rats administered tamoxifen, as well as salmon testis DNA that was modified in vitro with dG-Tam. Each of these samples gave tamoxifen-DNA adduct levels consistent with those previously reported (24, 27) (data not shown).

**32P-Postlabeling Analyses**

To confirm the results obtained by HPLC–ES-MS/MS, each of the human endometrial and breast DNA samples was assessed by \(^{32}\)P-postlabeling analyses. As a positive control, a DNA sample from the liver of a male F344 rat that had received 45 mg/kg of tamoxifen daily by gavage for 14 days (30) was also analyzed. When analyzed by method A, the rat liver sample gave rise to one major and several minor spots by TLC (data not shown) and to a single major peak by HPLC that eluted at 26 minutes (Fig. 2, A). This HPLC peak contained both dG-Tam and dG-desMeTam, which co-eluted in a single peak under the HPLC solvent gradient conditions used. Similar results were obtained when the 32P-postlabeling procedures we used. However, Orton and Topham (20) have noted that the chromatograms shown by Hemminki et al. (13) contained high levels of background radioactivity, which could account for some of their findings. In addition, Hemminki et al. did not use synthetic DNA adduct standards for comparison. By contrast, our HPLC–ES-MS/MS analyses included a well-characterized deuterated adduct standard to facilitate adduct identification and quantitation.

**DISCUSSION**

In this study we used HPLC–ES-MS/MS and \(^{32}\)P-postlabeling analyses to assess the presence of dG-Tam and dG-desMeTam in endometrial DNA from women who had been treated therapeutically with tamoxifen. Neither adduct was detected by either assay, even though the detection limits were two adducts per \(10^9\) nucleotides for dG-Tam and two adducts per \(10^8\) nucleotides for dG-desMeTam when the adducts were assessed by HPLC–ES-MS/MS and one adduct per \(10^9\) nucleotides when the adducts were measured by \(^{32}\)P-postlabeling.

Our results differ from those published by Hemminki et al. (13), who were the first to report the detection of tamoxifen-DNA adducts, at a level of 2.7 adducts per \(10^7\) nucleotides in endometrial DNA samples from women treated with tamoxifen. This adduct level is near the limit of detection of our HPLC–ES-MS/MS method but clearly within the limit of sensitivity of the \(^{32}\)P-postlabeling procedures we used. However, Orton and Topham (20) have noted that the chromatograms shown by Hemminki et al. (13) contained high levels of background radioactivity, which could account for some of their findings. In addition, Hemminki et al. did not use synthetic DNA adduct standards for comparison. By contrast, our HPLC–ES-MS/MS analyses included a well-characterized deuterated adduct standard to facilitate adduct identification and quantitation.

Our results also differ from those of Shibutani et al., who, using an improved \(^{32}\)P-postlabeling procedure coupled with HPLC separation, reported the presence of tamoxifen-DNA adducts in eight of 16 endometrial tissue samples from tamoxifen-treated women (14, 15). The adducts were identified on the basis of co-chromatography with synthetic standards, and in seven of the eight positive samples, dG-Tam was estimated to occur at 1.6–18 adducts per \(10^8\) nucleotides. The presence of dG-Tam at these levels in the samples we analyzed would
Endometrial DNA from a woman treated with tamoxifen and analyzed by method A.

Endometrial DNA from an untreated woman, analyzed by method A.

(14,15)

Another concern regarding the data reported by Shibutani et al. pertains to the configuration of the adducts detected. The predominant peaks detected by Shibutani et al. in several of the human endometrial DNA samples co-eluted with dG-Tam adduct standards that had a Z configuration. This finding differs from what has been observed in experimental animals (21–23) and in in vitro reactions (25,26), where the adducts have predominantly an E configuration. Furthermore, even when the major dG-Tam adducts detected by Shibutani et al. had an E configuration, the ratio of epimers varied substantially among the samples, which again is a result not obtained in experimental animals (21–23). Although the reasons for the high percentage of adducts with a Z configuration and the unusual ratio of epimers are not known, it is possible that the multiple enrichment and enzymatic steps involved in the 32P-postlabeling methodology used by Shibutani et al. could increase the likelihood of obtaining spurious results. The HPLC–ES-MS/MS technique we used has considerably fewer manipulations than 32P-postlabeling and, when we analyzed identical DNA samples from tamoxifen-treated cynomolgus monkeys by HPLC–ES-MS/MS, the results were very similar to those obtained by 32P-postlabeling (23) and chemiluminescence immunoassay (27).

An additional method that has been used to detect tamoxifen–DNA adducts is accelerator mass spectrometry (16). Although this method can detect as few as approximately two tamoxifen–DNA adducts per 1010 nucleotides (16), a detection limit substantially better than the ones we report for the HPLC–ES-MS/MS or 32P-postlabeling methods, the identity of the adducts was not established, and it is possible that the material being measured by accelerator mass spectrometry was non-covalently bound tamoxifen metabolites, as opposed to tamoxifen–DNA adducts.

In addition to HPLC–ES-MS/MS, we used two different 32P-postlabeling methods to assess tamoxifen–DNA adducts in the endometrial DNA samples. Method A complies with recommended protocols for 32P-postlabeling (31) and has been shown to have a 32P-postlabeling efficiency greater than 90% with tamoxifen–DNA adducts (32). Because the efficiency of this procedure when applied to the analysis of human endometrial samples has been questioned (34), we also used method B, which was used by Shibutani et al. to detect the presence of tamoxifen–DNA adducts in human endometrium (14,15). We found that the 32P-postlabeling efficiency of tamoxifen–DNA adducts formed in rat liver did not differ between our method (method A) and method B. Furthermore, regardless of whether the 32P-postlabeling was conducted by method A or method B or whether the chromatography was performed by TLC or HPLC, we found no evidence for the presence of dG-Tam or dG-desMeTam in the human endometrial DNA samples.

In addition to dG-Tam and dG-desMeTam, other DNA adducts formed by tamoxifen metabolites such as 4-hydroxytamoxifen (35) and α-hydroxy-N,N-didesmethyltamoxifen (36) have been characterized. At present, our HPLC–ES-MS/MS methodology is not configured to detect tamoxifen–DNA adducts from 4-hydroxytamoxifen or α-hydroxy-N,N-didesmethyltamoxifen; however, there was no indication that these adducts were present in any of the endometrial DNA samples when they were assayed by 32P-postlabeling.

In conclusion, we found no evidence with either detection method (i.e., HPLC–ES-MS/MS or 32P-postlabeling) for the formation of tamoxifen–DNA adducts in women who had taken tamoxifen. These findings are consistent with our earlier results (17,18) and suggest that the initiation of endometrial cancer by tamoxifen is not mediated by a genotoxic mechanism involving the formation of dG-Tam or dG-desMeTam.

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