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Identification of Tamoxifen-DNA Adducts in Monkeys Treated with Tamoxifen[1]

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ABSTRACT

The risk of developing endometrial cancer is increased in breast cancer patients treated with tamoxifen (TAM) and in healthy women undergoing TAM chemoprevention. We have detected previously TAM-DNA adducts in the endometrium of women receiving TAM (Shibutani *et al.*, Carcinogenesis, *21:* 1461–1467, 2000). To investigate the genotoxic damage induced by TAM in the uterus and other tissues of primates, we gave adult female cynomolgus monkeys six times the human-equivalent dose of TAM (2 mg/kg body weight/day) for 30 days. DNA samples were prepared from the uterus, ovary, liver, kidney, and brain cortex of three TAM-exposed monkeys and one control monkey and were analyzed as coded specimens. To identify the TAM-DNA adducts, we established a new high-performance liquid chromatography gradient system for ^{32}P-postlabeling/high-performance liquid chromatography analysis, which can resolve the *trans*- and *cis*-diastereoisomers of α-(N^2-deoxyguanosinyl)TAM (dG-N^2-TAM), α-(N^2-deoxyguanosinyl)-*N*-desmethylTAM, and α-(N^2-deoxyguanosinyl-)tamoxifen *N*-oxide. *Trans*-forms of dG-N^2-TAM and dG-N^2-*N*-desTAM adducts were detected in the livers of all three TAM-fed monkeys at levels of 2.7 adducts/10^8 nucleotides and 1.7 adducts/10^8 nucleotides, respectively. The levels of dG-N^2-TAM adducts observed in the uterus of one monkey and in the ovaries of two monkeys were ~10-fold lower than those observed in the livers. TAM exposure also induced dG-N^2-TAM adduct in the brain cortex of all three monkeys with a value of 1.5 adducts/10^8 nucleotides. No TAM-DNA adducts were detected in the kidneys or in any tissues obtained from the unexposed monkey. Our results suggest that women receiving TAM may form genotoxic damage in many organs, including the reproductive organs.

INTRODUCTION

TAM[3] is widely used as endocrine antiestrogen therapy for breast cancer patients and as a chemopreventive agent for healthy women at high risk for breast cancer (1, 2). However, treatment with TAM is associated with an increased incidence of endometrial cancer in breast cancer patients (3, 4) and in healthy women aged ≥50 years who were enrolled in the Breast Cancer Prevention Trial initiated by the National Surgical Adjuvant Breast and Bowel Project (2). TAM has been classified as a human carcinogen by the International Agency for Research on Cancer (5).

p.o. exposure of TAM induces hepatocellular tumors in rats (6), which were associated with the formation of covalent DNA adducts induced by the activated metabolites of the drug (7–9). It is known that TAM is converted by Phase I enzymes to several reactive species, including α-OHTAM, *N*-desTAM, TAM *N*-oxide, and 4-OHTAM (Fig. 1). α-Hydroxylation of these metabolites, followed by *O*-sulfonation and/or *O*-acetylation, constitutes a major pathway capable of forming DNA adducts (8, 10). In fact, α-OHTAM is sulfonated by rat and human hydroxysteroid sulfotransferases (11, 12) and reacts with the exocyclic amino group of guanine in DNA, forming two *trans* (fr-1 and fr-2) and two *cis* (fr-3 and fr-4) diastereoisomers of dG-N^2-TAM (Fig. 1; Refs. 8 and 10). Mass-spectroscopic and ^{32}P-postlabeling/HPLC analyses demonstrated that dG-N^2-TAM and dG-N^2-*N*-desTAM are major hepatic DNA adducts in rodents exposed to TAM (13, 14). α-(N^2-deoxyguanosinyl)tamoxifen *N*-oxide was also detected as a minor adduct in the livers of mice treated with TAM. The three TAM-DNA adducts account for >95% of hepatic DNA adducts induced by TAM (14). TAM-DNA adducts display a high mutagenic potential in mammalian cells (15) and in the liver of λ/*lac I* transgenic rats treated with TAM (16). If TAM-DNA adducts are not repaired (17), mutations may occur at adducted sites and initiate the development of cancer.

Several laboratories have attempted to detect TAM-DNA adducts in breast cancer patients receiving TAM therapy. Low levels of a putative DNA adduct (2.7–5.5 adducts/10^9 dNs) were detected in human leukocytes and endometrium by Hemminki *et al.* (18, 19). However, the formation of TAM-DNA adducts in human leukocytes and endometrium was not detected by Phillips and Carmichael *et al.* (20, 21). By minimizing the high background observed in previous reports (18–21), thereby increasing the sensitivity of the ^{32}P-postlabeling analysis, we were able to detect TAM-DNA adducts (total, 0.2–18 adducts/10^8 dNs) in the endometrium from 8 of 16 breast cancer patients (22). Umemoto *et al.* (23) have also detected a low level of TAM-DNA adducts (1.5 ± 0.6 adducts/10^9 dNs) in leukocytes from 6 of 47 breast cancer patients using a ^{32}P-postlabeling/HPLC analysis.

Using a newly developed modification of the ^{32}P-postlabeling/HPLC analysis, we report here the presence of significant amounts of TAM-DNA adducts in the liver, uterus, ovary, and brain cortex of cynomolgus monkeys dosed p.o. for 30 days with 2 mg of TAM/kg bw/day.

MATERIALS AND METHODS

Chemicals. [γ-^{32}P]ATP (specific activity, 6000 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, IL). Polyethylenimine-cellulose plates were purchased from Machery-Nagel (Duren, Germany). TAM, proteinase K, potato apyrase, and nuclease P1 were purchased from Sigma (St. Louis, MO) and Boehringer Mannheim (Indianapolis, IN), respectively. RNase A, RNase T1, micrococcal nuclease, and spleen phosphodiesterase were obtained from Worthington Biochemical Co. (Freehold, NJ).

DNA Extraction from Monkey Tissues. The subjects of this study were 4 adult (19 years old) retired breeder female cynomolgus (*Macaca fascicularis*) monkeys housed and treated at Corning Hazelton Laboratories (Vienna, VA). Animal care was provided at Hazleton Laboratories in accordance with the standards established by the Association for Assessment and Accreditation for Laboratory Animal Care. The experimental protocols were approved by the Hazleton Animal Care and Use Committee. TAM (1 mg/kg bw) was administered 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 the rate of 2 ml/kg bw/dose for a total daily dose of 2 mg of TAM/kg bw. The exposure lasted for 30 days. The animals were euthanized, and the organs (brain cortex,

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[3] The abbreviations used are: TAM, tamoxifen; dN, 2′-deoxynucleoside; HPLC, high-performance liquid chromatography; dG, 2′-deoxyguanosine; $dG_{3'p}$, 2′-deoxyguanosine 3′-monophosphate; TAM *N*-oxide, tamoxifen *N*-oxide; OHTAM, hydroxytamoxifen; dG-N^2-TAM, α-(N^2-deoxyguanosinyl)tamoxifen; dG-N^2-*N*-desTAM, α-(N^2-deoxyguanosinyl)-*N*-desmethyltamoxifen; bw, body weight.

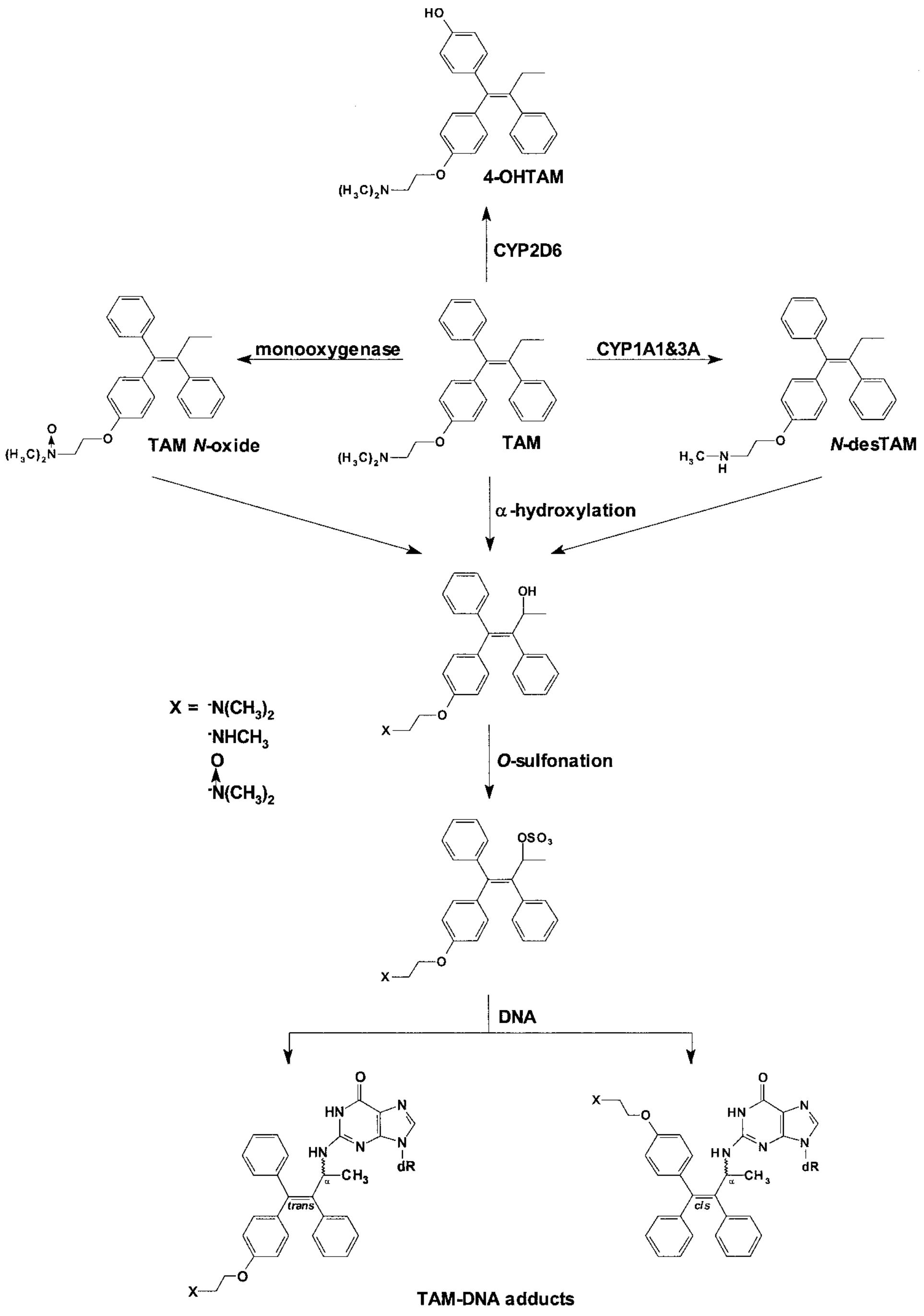

Fig. 1. Formation of TAM-DNA adducts via α-hydroxylation of TAM metabolites.

liver, kidney, ovary, and uterus) were harvested for DNA isolation. DNA was isolated by nonorganic extraction (Stratagene, La Jolla, CA, or Qiagen, Valencia, CA), followed by digestion with 1 unit/ml amyloglucosidase in the case of DNA isolated from liver tissue. DNA was quantified by UV spectroscopy. The concentration of the DNA was estimated as $50\ \mu g = A_{260\ nm}1$. DNA samples from the uterus, ovary, liver, kidney, and brain cortex were analyzed as coded specimens.

Digestion of DNA Samples. Ten micrograms of DNA were digested at 37°C for 2 h in 30 μl of 17 mM sodium succinate buffer (pH 6.0) containing 8 mM $CaCl_2$, using 15 units of micrococcal nuclease and 0.15 unit of spleen phosphodiesterase. Subsequently, 1 unit of nuclease P1 was added, and the reaction mixture was incubated at 37°C for 1 h. Samples were dissolved in 100 μl of distilled water and extracted twice with 200 μl of butanol. The butanol fraction was back extracted with 50 μl of distilled water, dried, and then used for analysis of TAM-DNA adducts. Approximately 95% of TAM-DNA adducts were recovered by butanol extraction.

Determination of ^{32}P-labeled DNA Adducts by HPLC. Digests in pooled extracts were labeled with ^{32}P and developed for 16 h on a 10 × 10 cm of polyethylenimine-cellulose thin-layer plate using 2.3 M sodium phosphate buffer (pH 6.0), with a paper wick (22). ^{32}P-labeled products remaining on the TLC plate were recovered, using 4 M pyrimidinium formate (pH 4.3), and evaporated to dryness. Recovery of ^{32}P-labeled products was ~84%. The ^{32}P-labeled products were subjected to a Hypersil BDS C_{18} analytical column (0.46 × 25 cm, 5 μm; Shandon), eluted at a flow rate of 1 ml/min with a linear gradient of 0.2 M ammonium formate and 20 mM H_3PO_4 (pH 4.0), containing 20–30% or 18–30% acetonitrile for 40 min, 30–50% acetonitrile for 5 min, followed by an isocratic condition of 50% acetonitrile for 15 min. The radioactivity was monitored by radioisotope detector (Berthold LB506 C-1; ICON Scientific Inc.) linked to a Waters 990 HPLC instrument. Standard stereoisomers of $dG_{3'p}$-N^2-TAM (11), $dG_{3'p}$-N^2-N-desTAM (24), and $dG_{3'p}$-N^2-TAM N-oxide (25) were prepared by methods described in previous publications and labeled with ^{32}P.

The relative adduct levels were calculated as described previously (25). When an oligodeoxynucleotide containing a single dG-N^2-TAM adduct was used as a standard for ^{32}P-postlabeling analysis, the recovery of TAM-DNA adducts was 56% (26). Therefore, the actual level of TAM-DNA adducts was estimated by dividing the experimental values by 56%. The baseline of ^{32}P on ^{32}P-postlabeling/HPLC analysis varied depending on tissues used; the detection limits were $\sim 0.8 \times 10^{-9}$ adducts for uterine and ovarian samples, 1.5×10^{-9} adducts for kidney and brain cortex samples, and 4×10^{-9} adducts for liver samples, respectively.

RESULTS

We have developed an HPLC gradient system for a ^{32}P-postlabeling/HPLC analysis that resolves *trans*- and *cis*-diastereoisomers of $dG_{3'P}$-N^2-TAM, $dG_{3'P}$-N^2-*N*-desTAM, and $dG_{3'P}$-N^2-TAM *N*-oxide adducts. As shown in Fig. 2*A*, standards of *trans*- (fr-1 and fr-2) and *cis*- (a mixture of fr-3 and fr-4) isomers of $dG_{3'P}$-N^2-TAM, $dG_{3'P}$-N^2-*N*-desTAM, and $dG_{3'P}$-N^2-TAM *N*-oxide can be resolved in 55 min. Using this procedure, DNA from the uterus, ovary, liver, kidney, and brain cortex of three TAM-fed monkeys and one unexposed monkey were analyzed to determine TAM-DNA adducts. Samples were coded during the analysis, after which, the TAM status was revealed.

When uterine and ovarian DNA samples (10 μg) were analyzed, fr-2 of the *trans*-$dG_{3'P}$-N^2-TAM adduct was detected in the one uterine and two ovarian DNA samples (Table 1). The level of uterine TAM adduct in F15 was 0.52 adducts/10^8 dNs (Fig. 2*B*); TAM adducts were not detected in the ovary of this monkey. $dG_{3'P}$-N^2-TAM adducts were also observed in the ovaries of two monkeys, F4 (0.2 adducts/10^8 dNs) and F14 (0.42 adducts/10^8 dNs). TAM-DNA adducts were not detected in the uterus and ovary of the untreated control monkey (Fig. 2*C*).

Table 1 *TAM-DNA adducts in monkey tissues*[a]

Organ	dG-N^2-TAM	dG-N^2-*N*-desTAM	Total
	(adducts/10^8 dNs)		
Uterus			
F7 (control)	N.D.[b]	N.D.	N.D.
F4 (TAM)	N.D.	N.D.	N.D.
F14 (TAM)	N.D.	N.D.	N.D.
F15 (TAM)	0.52 ± 0.23	N.D.	0.52
Ovary			
F7 (control)	N.D.	N.D.	N.D.
F4 (TAM)	0.20	N.D.	0.20
F14 (TAM)	0.42 ± 0.06	N.D.	0.42
F15 (TAM)	N.D.	N.D.	N.D.
Liver			
F7 (control)	N.D.	N.D.	N.D.
F4 (TAM)	2.89 ± 0.58	1.89 ± 0.47	4.78
F14 (TAM)	2.84 ± 0.59	1.64 ± 0.51	4.48
F15 (TAM)	2.44 ± 0.17	1.60 ± 1.00	4.04
Total	2.72 ± 0.25	1.71 ± 0.16	4.43 ± 0.37
Kidney			
F7 (control)	N.D.	N.D.	N.D.
F4 (TAM)	N.D.	N.D.	N.D.
F14 (TAM)	N.D.	N.D.	N.D.
F15 (TAM)	N.D.	N.D.	N.D.
Brain cortex			
F7 (control)	N.D.	N.D.	N.D.
F4 (TAM)	2.48 ± 0.35	N.D.	2.48
F14 (TAM)	0.73 ± 0	N.D.	0.73
F15 (TAM)	1.15 ± 0.28	N.D.	1.15
Total	1.45 ± 0.91	N.D.	1.45 ± 0.91

[a] Data are expressed as mean values ± SD from two to three samples.
[b] N.D., not detected.

Interestingly, *trans*-$dG_{3'P}$-N^2-TAM (fr-2; 1.45 ± 0.91 adducts/10^8 dNs) was detected in the brain cortex of all TAM-treated monkeys (Table 1). When brain sample F4 (2.48 adducts/10^8 dNs; Fig. 2*E*) was coinjected with ^{32}P-labeled authentic standards (Fig. 2*D*), the product

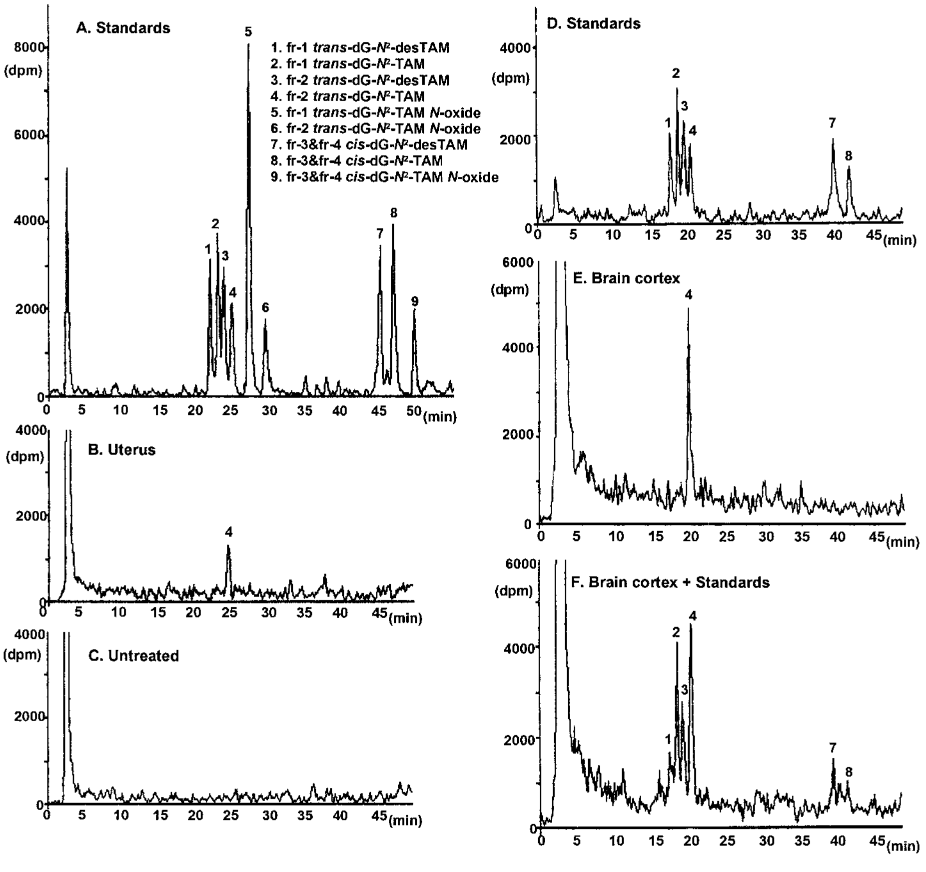

Fig. 2. ^{32}P-postlabeling/HPLC analysis of TAM-DNA adducts in monkey uterus and brain cortex. In *A*, standards containing stereoisomeric *trans*- and *cis*-forms of ^{32}P-labeled dG-N^2-TAM, dG-N^2-*N*-desTAM, and α-(N^2-deoxyguanosinyl)tamoxifen *N*-oxide were subjected to a Hypersil BDS C_{18} analytical column (0.46 × 25 cm, 5 μm), eluted at a flow rate of 1 ml/min with a linear gradient of 0.2 M ammonium formate and 20 mM H_3PO_4 (pH 4.0), containing 20–30% acetonitrile for 40 min, and 30–50% acetonitrile for 5 min, followed by an isocratic condition of 50% acetonitrile for 15 min. The radioactivity was monitored by radioisotope detector linked to the HPLC instrument. Uterine DNA samples (10 μg) from TAM-treated (*B*; F15) and untreated (*C*; F7) monkeys were digested by enzymes, labeled with ^{32}P, partially purified using TLC plate, and analyzed by HPLC/radioisotope detector, as described in "Materials and Methods." In *D*, standards containing diastereoisomers of dG-N^2-TAM and dG-N^2-*N*-desTAM (*D*) were subjected to HPLC with an on-line radioisotope detector. In *E*, brain cortex DNA sample (F4; 10 μg) was analyzed by ^{32}P-postlabeling/HPLC analysis. *F*, cochromatography of brain cortex sample (*E*) with standards (*D*).

was identified as $dG_{3'p}$-N^2-TAM (fr-2; Fig. 2*F*). DNA adducts were not detected in brain tissue from the untreated control (Table 1).

When hepatic DNA samples were analyzed, the baseline of ^{32}P on ^{32}P-postlabeling/HPLC chromatography was much higher than that obtained from other tissues. Although the hepatic DNA samples were repurified using our protocol (11), the baseline was not significantly reduced. Therefore, the adduct detection limit (4×10^{-9} adducts) was three to five times higher than that for the other tissues. Two TAM-DNA adducts predominated in all TAM-treated monkeys (Fig. 3, *A–C*). These were fr-2 of *trans*-$dG_{3'p}$-N^2-TAM (2.72 ± 0.25 adducts/10^8 dNs) and fr-2 of *trans*-$dG_{3'p}$-N^2-*N*-desTAM (1.71 ± 0.16 adducts/10^8 dNs; Table 1). By coinjecting the F14 sample with ^{32}P-labeled authentic standards, these products were identified as $dG_{3'p}$-N^2-TAM (fr-2) and $dG_{3'p}$-N^2-*N*-desTAM (fr-2) adducts (data not shown). The mean level of TAM-DNA adducts in the monkey livers was 4.43 ± 0.37 adducts/10^8 dNs, whereas no adducts were found in the control hepatic DNA sample (Fig. 3*D*). TAM-DNA adducts were not detected in any of the kidney DNA samples (Table 1).

DISCUSSION

We have developed an HPLC gradient system that can resolve diastereoisomers of ^{32}P-labeled $dG_{3'p}$-N^2-TAM, $dG_{3'p}$-N^2-*N*-desTAM, and $dG_{3'p}$-N^2-TAM *N*-oxide in 55 min. Using a sensitive ^{32}P-postlabeling/HPLC analysis, we determined the level of TAM-DNA adducts in tissues from monkeys given TAM (2 mg/kg bw/day) for 30 days. Interestingly, $dG_{3'p}$-N^2-TAM adducts were detected in the uterus of one monkey and in the ovary of two monkeys. Thus, TAM-DNA adducts were formed in the reproductive organs of some, but not all, monkeys, as has been observed in the endometrium of women taking TAM (22). In contrast, TAM-DNA adducts were not detected in uterus of rats treated with TAM (27), suggesting that the formation of TAM-DNA adducts may be species specific. Our studies suggest that the monkey is a suitable species to predict genotoxicity of this antiestrogen in humans.

Surprisingly, $dG_{3'p}$-N^2-TAM adducts were detected in the brain cortex of all three monkeys given TAM, at a level only three times lower than that observed in the liver. Sulfonation of dehydroepiandrosterone, a substrate of hydroxysteroid sulfotransferase, has been observed in human fetal brain slices. More recently, hydroxysteroid sulfotransferase was identified as a neurosteroid sulfotransferase in rat brain (28, 29). Such enzymes are likely to be involved in the formation of TAM-DNA adducts in the monkey brain.

The $dG_{3'p}$-N^2-TAM and $dG_{3'p}$-N^2-*N*-desTAM adducts were also detected in the livers of all three monkeys treated with TAM; the level of total TAM-DNA adducts was 4.43 ± 0.37 adducts/10^8 dNs. High levels of TAM-DNA adducts were detected in the livers of rats and mice treated with a high dose of TAM (14). When rats and mice were treated p.o. with 45 mg of TAM/kg bw/day and 120 mg of TAM/kg bw/day, respectively, for 7 days, the hepatic TAM-DNA adduct levels were 216 adducts/10^8 dNs and 56 adducts/10^8 dNs in rats and mice, respectively (14). The daily TAM doses given to rats and mice were 23- and 60-fold, respectively, higher than that for monkeys (2 mg/kg bw/day). Therefore, if rats and mice are treated with the dose used for monkeys, the levels of hepatic TAM-DNA adducts should be much

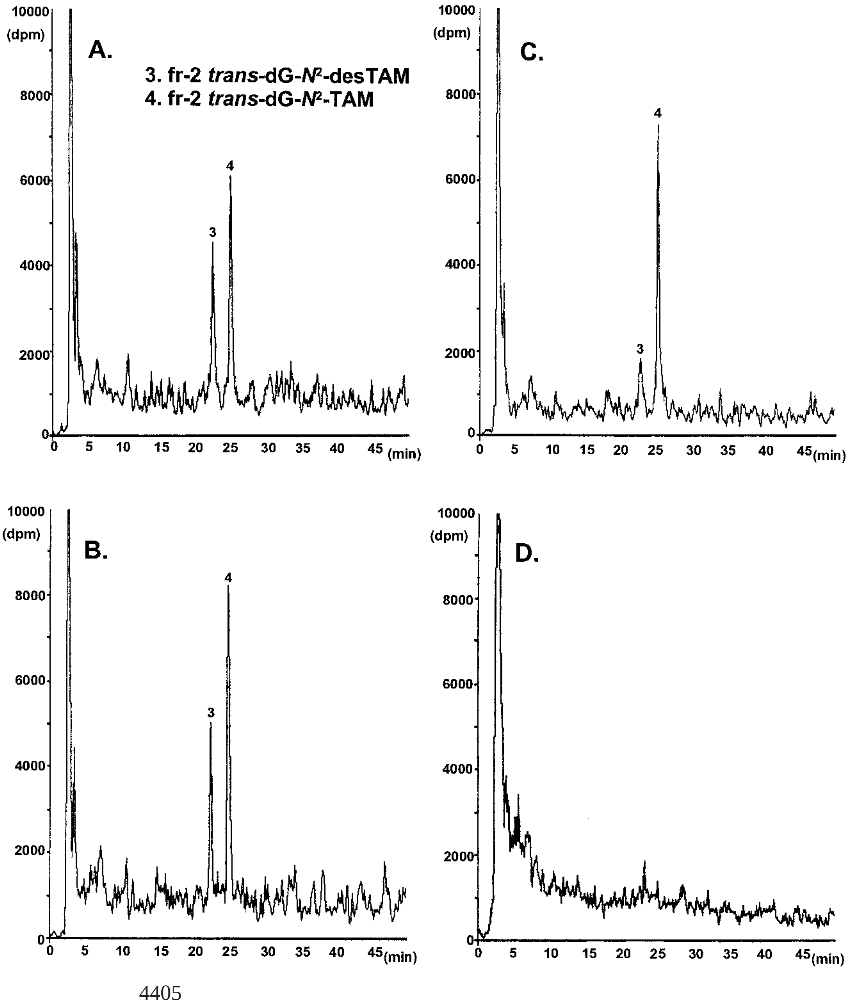

Fig. 3. ^{32}P-postlabeling/HPLC analysis of TAM-DNA adducts in monkey liver. Hepatic DNA samples (10 μg) from TAM-treated monkey (*A*, F4; *B*, F14; *C*, F15) and untreated monkey (*D*, F7) were analyzed by ^{32}P-postlabeling/HPLC analysis, as described in the legend to Fig. 2 using 18–30% acetonitrile, instead of 20–30% acetonitrile, for HPLC gradient condition.

lower than those observed at higher doses of TAM. In only rats, hepatocarcinoma is promoted (6). This may be attributable to the fact that TAM-DNA adducts have a long half-life in rat liver (7, 30), whereas the adducts are rapidly repaired in mouse liver (31). Additional analyses are required to determine the species specificity of hepatic TAM-DNA adduct formation and repair.

Using the same monkey DNA samples analyzed in our study, the levels of TAM-DNA adducts in the uterus, liver, and brain cortex have been determined using a TAM-DNA chemiluminescence immunoassay and HPLC electrospray tandem mass spectrometry.[4] The values obtained using the other methods were strikingly similar to those reported here. Therefore, p.o. TAM exposure induces DNA damage in the liver, brain, and female reproductive organs of primates. TAM-DNA adducts were not detected in the livers of women treated with TAM (32). Methodological differences in enzyme digestion of DNA samples and/or labeling of adducted nucleotides with ^{32}P (33) may be responsible for the inability to detect liver TAM-DNA adducts. On the other hand, it is possible that the daily TAM dose given to women, which is ~6-fold lower than the daily doses given here to cynomolgus monkeys, is insufficient for the formation of hepatic TAM-DNA adducts. Additional clinical studies are needed to evaluate the genotoxic risk of TAM in humans.

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