Tamoxifen-DNA Adduct Formation in Rat Liver Determined by Immunoassay and $^{32}$P-Postlabeling

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ABSTRACT

Tamoxifen (TAM), a nonsteroidal antiestrogen used as a chemotherapeutic and chemopreventive agent for breast cancer, induces liver tumors in rodents and covalent DNA adduct formation in hepatic DNA. Here, we report the development and validation of highly sensitive and specific immunoassays for the determination of TAM-DNA adducts. Rabbits were immunized with calf thymus DNA, chemically modified with α-acetyltamoxifen to 2.4 adducts per 100 nucleotides, and the resulting antisera were characterized by competitive dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) and chemiluminescence immunoassay (CIA). Compared with DELFIA, the CIA has a much lower background and a 20-fold increase in sensitivity. For TAM-DNA modified in vitro, 50% inhibition was at 2.0 ± 0.11 (mean ± SE, n = 18) fmol of (E)-α-(N2-deoxyguanosinyl)tamoxifen (TAM-dG) adduct in TAM-DNA by DELFIA. For TAM-DNA modified to 4.8 adducts in 10⁶ nucleotides, 50% inhibition was at 20.6 ± 6.6 (mean ± SE, n = 8) fmol of TAM-dG in TAM-DNA by DELFIA and at 0.92 ± 0.11 (mean ± SE, n = 10) fmol of TAM-dG in TAM-DNA by CIA. No inhibition was observed in either assay with up to 20 μg (62.5 nmol of nucleotides) of unmodified DNA. The individual adducts TAM-dG and (Z)-α-(N²-deoxyguanosinyl)tamoxifen and the individual compounds TAM and 4-OH-TAM gave DELFIA 50% inhibitions at 828, 2229, 5440, and 8250 fmol, respectively. For assay validation, TAM-dG levels were determined by DELFIA, CIA, and $^{32}$P-postlabeling in TAM-DNA samples modified in vitro to different levels, and comparable values were obtained in all three assays. Further validation was obtained in vivo in rat liver. DNA adducts of TAM were measurable in rat liver 24 h after a single i.p. dose of 45 mg TAM/kg body weight and after daily p.o. dosing for 7 days with 5.0, 10.0, and 20.0 mg TAM/kg body weight. In addition, TAM-DNA adducts disappeared over 21 days in rats on a control diet that were first given p.o. TAM at 45 mg/kg/day for 4 days. In the rat experiments, TAM-DNA adduct levels determined by CIA compared well with those determined by $^{32}$P-postlabeling, although the CIA gave an underestimate at the highest doses. For rat liver samples, the detection limit by CIA was 3 adducts per 10⁷ nucleotides (0.2 fmol of adducts per 20 μg of DNA).

INTRODUCTION

TAM² is widely used as a chemotherapeutic drug for treating breast cancer, and it was recently approved as a prophylactic agent for preventing breast cancer, with a standard therapy duration of ≥5 years (1). In addition, TAM was evaluated in 13,888 healthy women at high risk for breast cancer in the Breast Cancer Prevention Trial, in which a dose of 20 mg/day TAM for 3 years decreased the incidence of breast cancer by ~50% (2). Furthermore, TAM use has been associated with a ~35% decrease in incidence of osteoporotic bone fractures (3) and a small but significant increase in the incidence of endometrial cancer (2, 4–7) and pulmonary embolism (2). In rats, chronic p.o. exposure to TAM has induced high incidences of hepatic adenomas and hepatocellular carcinomas (8–12). Consistent with tumorigenesis are findings of hepatic aneuploidy and mitotic spindle disruption (13, 14) as well as DNA adduct formation in rat liver and kidney (15, 16). In mice, TAM exposure produced proliferative lesions in oviduct and uterus (17), followed by uterine carcinoma (18). In rodents, the weight of evidence suggests that TAM may be carcinogenic, at least partially, through genotoxic mechanisms. However, testing for TAM-induced mutagenicity in the Ames test was negative (5, 19), and the chromosomal aberration assay performed in vitro in human cells (5) showed no aberrations. In addition, there is some controversy regarding the formation of TAM-DNA adducts in human tissues (20–27), it is not clear that the mechanisms elucidated in rodent models are directly applicable to the human.

To understand the potential role of TAM-DNA adducts in induction of genetic alterations and cancer, it is necessary to monitor the formation and persistence of these adducts in susceptible target tissues of exposed animals and humans. The $^{32}$P-postlabeling assay, currently in widespread use for measuring TAM-DNA adduct levels (16, 28–30), is excellent for in vitro modified DNA samples and cultured cells or tissues of animals exposed to a single agent. Unfortunately, because this assay is not specific and cannot select only the adduct of interest, it, therefore, produces equivocal results when applied to human samples, in which complex chemical exposures occur and multiple different adducts are present. In response to the need for a specific TAM-DNA adduct assay, polyclonal antisera were elicited against highly modified TAM-DNA, and two highly sensitive and specific immunoassays, with different end points, were developed. Validation of these assays and their application in the measurement of TAM-DNA adducts in rat liver are presented here.

MATERIALS AND METHODS

Chemicals. TAM, CT DNA, tricaprylin, and casein were obtained from Sigma Chemical Co. (St. Louis, MO). Proteinase K, RNase A, and RNase T1 were from Boehringer Corp. (Lewes, Sussex, United Kingdom). Water-saturated phenol was from Rathburn Chemicals (Peebleshire, United Kingdom). Low-fluorescence 96-well microtiter plates for DELFIA, Eu-labeled streptavidin, and enhancement solution were from Wallac (Gaithersburg, MD). Biotinylated fluorescence 96-well microtiter plates for DELFIA, Eu-labeled streptavidin, and enhancement solution were from Wallac (Gaithersburg, MD). Biotinylated antirabbit IgG used for the DELFIA was obtained from Vector Laboratories (Burlingame, CA). Opaque 96-well plates for CIA were purchased from Corning Costar Corp. (Cambridge, MA). Reagents used in the CIA including biotinylated antirabbit IgG, streptavidin-alkaline phosphatase, IgG, and CDP-Star with Emerald II were obtained from Tropix (Bedford, MA). All other chemicals used were of analytical grade from commercial sources.

Preparation of Immunogen. TAM-DNA was prepared as described previously (16). Briefly, α-acetyltamoxifen (12 mg) in ethanol (20 ml) was added to CT DNA (50 mg) in water (40 ml), and the mixture was incubated at 37°C for 4 h. After addition of 1 g of sodium acetate, the free α-hydroxytamoxifen was removed by extraction 7 times with 20 ml of ethyl ether. The TAM-DNA was subsequently precipitated with 2.5 volumes of ethanol; washed; dried; resuspended in sterile, distilled deionized water; sheared with a 20-gauge needle; and stored at −20°C until use. The resulting TAM-DNA, used as the immunogen, was found to be modified to 2.4 adducts per 100

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2 The abbreviations used are: TAM, tamoxifen; CT, calf thymus; DELFIA, dissociation-enhanced lanthanide fluoroimmunoassay; CIA, chemiluminescence immunoassay; HPLC, high-performance liquid chromatography; bw, body weight; PBST, PBS with 0.05% Tween 20; TAM-dG, (E)-α-(N²-deoxyguanosinyl)tamoxifen.
nucleotides by UV-Vis spectroscopy (using molar absorption coefficient of 19,000 at 270 nm in a 1-cm path length cuvette) after hydrolysis and HPLC purification. TAM-DNA samples modified to −0.1–3880 adducts/10^9 nucleotides were obtained by using essentially the same protocol and varying the concentration of α-acetoxytamoxifen or the duration of incubation. The modification levels were estimated using UV-Vis spectroscopy after hydrolysis and adduct separation by HPLC for highly modified TAM-DNA samples or 32P-postlabeling for low modified TAM DNA samples (16).

**Immunization.** Three female New Zealand White rabbits (5 months old), purchased from Covance (Denver, PA), were immunized with TAM-DNA electrostatically coupled to methylated BSA. For the initial immunization, TAM-DNA (0.46 mg), methylated BSA (0.46 mg) in 0.16 M NaCl, and Freund’s complete adjuvant were emulsified and injected i.m. into each hind leg of each rabbit per injection. The rabbits were immunized with the same amounts of TAM-DNA and methylated BSA emulsified in Freund’s incomplete adjuvant once per week for 3 additional weeks and again at 6 weeks. The blood was collected in glass tubes before immunization, at week 5, and weekly between weeks 8 and 16. The blood was allowed to clot and the serum was removed and stored in aliquots at −20°C until use.

**Administration of TAM to Rats and Liver DNA Extraction.** Female Fischer 344 rats were bred on site and were allowed access to food and water ad libitum. Rats were 6 weeks old (120–130 g) at the start of treatment. For the single-dose study, groups of four animals received TAM dissolved in tricaprylin (0.10–0.15 ml), by i.p. injection, at a dose of either 4.5 or 45 mg/kg bw. Control rats received an equivalent dose of tricaprylin vehicle alone. The animals were euthanized by cervical dislocation 24 h after dosing.

For dosing up to 7 days, groups of four rats received TAM dissolved in tricaprylin (0.1–0.15 ml), by p.o. gavage, at 0, 0.5, 1.0, 5.0, 10.0, and 20.0 mg TAM/kg bw, whereas control animals received an equivalent amount of tricaprylin vehicle only. The rats were euthanized by cervical dislocation 24 h after the final TAM dose.

To study the persistence of adducts, we gave groups of four rats 45 mg TAM/kg bw by p.o. gavage for 4 days, followed by 21 days on control diet. Animals were euthanized on days 5, 7, 10, 14, and 21. Control animals in this experiment received equivalent doses of tricaprylin vehicle alone.

Livers were removed from the experimental animals and stored at −70°C until further processing. DNA was isolated from tissue by the method of Gupta (31) using proteinase K digestion, phenol/chloroform extraction, and ethanol precipitation, followed by digestion with RNase A and T1.

**Processing of DNA for Analysis.** Aliquots (1.0 ml) of DNA (100 μg) in PBS were sonicated for 15 s using a Sonifier Cell Disruptor (Heat Systems-Ultrasonics, Inc., Plainview, NY), heat-denatured at 90°C for 5 min, and centrifuged at 3000 rpm. Supernatants were saved for analysis of TAM-DNA adducts by immunoassays. For 32P-postlabeling analysis, aliquots of DNA (4 μg) were digested to nucleoside 3'-monophosphates with micrococcal nuclease (0.14 unit) and spleen phosphodiesterase (0.65 unit), followed by nuclease P1, as described previously (32, 33).

**DELFIA.** Microtitration plates were coated with 18 ng of DEAE-dextran in water (300 μl) at 4°C for 24 h and washed with three cycles of PBS using an automated plate washer (Ultra Wash Plus; Dynatech Laboratories, Gaithersburg, MD). Plates were coated with 660 μg of sonicated CT DNA or immunogen TAM-DNA (2.4% modified) in 0.1 ml of PBS and evaporated to dryness at 37°C. Plates were washed with three cycles of PBS 0.05% of NaN₃ and stored frozen until use.

During the execution of the DELFIA, after each incubation, plates were washed three times with PBST. Non-specific binding was reduced by incubating the wells with 300 μl casein (2.5 mg/ml) in PBST for 1 h at 37°C. Equal volumes (50 μl) of anti-TAM-DNA (diluted to 1:1500) in PBST and serially diluted TAM-DNA standard (modified either to 2.4% or to 4.8 adducts per nucleotide) or test sample in PBS were mixed and incubated at 37°C for 30 min prior to adding to the wells. The DNA content in the standard curve wells was maintained at 3 μg by addition of appropriate amounts of sonicated and denatured carrier CT DNA to normalize the matrix effect. The mixture containing TAM-DNA antibody and TAM-DNA standard or biological sample was transferred to the microtitration plate and incubated for 90 min. After washing, biotinylated antirabbit IgG (1:2500 dilution in PBST) was added and incubated for a further 90 min. After washing again, Eu-labeled streptavidin (100 μl, 1:2000 dilution in assay buffer; Wallac) was placed on the wells and incubated at room temperature for 1 h. Finally, the plates were washed, and 100 μl of enhancement solution (Wallac) was added to the wells. After a 1-h incubation at room temperature, the fluorescence was measured using the DELFIA 1234 Research Fluorometer.

**CIA.** Opaque microtitration plates were coated with DEAE-dextran, as described for the DELFIA, followed by 20 pg of unmodified CT-DNA or 2.4% modified TAM-DNA. Plates were coated, washed, and stored as described above for the DELFIA. The special reagents used in this assay were obtained from Tropix. To perform the CIA, we washed plates and blocked void spaces using 0.25% 1 Block in PBST. Equal volumes of antisera (diluted to 1:1,500,000 in PBST) and serially diluted TAM-DNA standard or biological sample were mixed and incubated for 30 min at 37°C. 100-μl aliquots of the mixtures were transferred to microtiter plate wells and incubated for 90 min at 37°C. After washing, biotinylated antirabbit IgG antibody (100 μl, 1:5000 dilution in PBST) was added and incubated at 37°C for 90 min. After washing, streptavidin-alkaline phosphatase (100 μl, 1:6000 in PBST) was added and incubated at room temperature for 60 min, followed by washing with three cycles of PBST and two cycles of Tris buffer (20 mm Tris-1 mm MgCl₂, pH 9.5). Finally, CDP-Star containing Emerald II enhancer (100 μl) was added and incubated at room temperature for 30 min. Light emission was measured at 542 nm using a TR717 Microplate Luminometer (PE Applied Biosystems, Foster City, CA).

**32P-Post labeling.** 32P-postlabeling was carried out as described previously (32), but with a new solvent system (16). Briefly, 4-μg aliquots of digested DNA were incubated with 50 μCi of [γ-32P]ATP (ICN Biomedicals, High Wycombe, United Kingdom; specific activity, 4000 Ci/mmol) and 6 units of polynucleotide kinase (Advanced Biotechnologies, Leatherhead, Surrey, United Kingdom). Resolution of the 32P-labeled adducts was then carried out on polyethyleneimine-cellulose TLC plates (Machery-Nagel, Camlab, Cambridge, United Kingdom) with the following solvents: D1, 2.3 M sodium phosphate (pH 5.8); D2, 2.28 M lithium formate and 5.2 M urea (pH 3.5); and D3, 0.52 M lithium chloride, 0.32 M Tris-HCl, and 5.2 M urea (pH 8.0). The DNA adducts were visualized by autoradiography of chromatograms at ~85°C and quantified from Cerenkov counting of the excised areas of the chromatograms. The radioactivity present in the adduct spots and the specific activity of the ATP (33) were used to calculate adduct levels, which were expressed as total adducts/10^8 nucleotides. The values obtained are based on labeling efficiency of ~90%.

**RESULTS**

**Characterization of the TAM-DNA Antiserum by Competitive Immunoassays.** TAM-DNA (2.4% modified), prepared by chemical modification of CT DNA with α-acetoxytamoxifen, was used to immunize rabbits and the resulting antiserum used to develop two immunoassays. Characterization of the TAM-DNA antiserum by both DELFIA and CIA showed that the sera from three rabbits contained high titers, allowing dilutions of 1:300,000 and 1:3,000,000 for DELFIA and CIA, respectively. Competitive DELFIAs were performed to elucidate antibody specificity (Table 1). In addition to

<table>
<thead>
<tr>
<th>Sample</th>
<th>TAM-DNA (immunogen)</th>
<th>TAM-DNA (low modified)</th>
<th>α,4-Diaceotoxytamoxifen-DNA</th>
<th>α,4-Dihydroxytamoxifen-DNA</th>
<th>α-Acetoxydioxifene-DNA</th>
<th>TAM-DG</th>
<th>(Z)-α-[N²-deoxyguanosinoyl]tamoxifen</th>
<th>TAM</th>
<th>4-OH TAM</th>
<th>Unmodified DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of adducts/no. of nucleotides</td>
<td>2.4/100</td>
<td>4.8/10⁶</td>
<td>0.4/100</td>
<td>0.4/100</td>
<td>0.18/100</td>
<td>827.5</td>
<td>8229.0</td>
<td>5400.0</td>
<td>8250.0</td>
<td>180 μg/g</td>
</tr>
<tr>
<td>DIELFA (fmol TAM equivalent at 50% inhibition)</td>
<td>2.0 ± 0.11 (n = 18)</td>
<td>20.6 ± 6.60 (n = 8)</td>
<td>24.6 ± 1.20</td>
<td>522.0 ± 16.37</td>
<td>ND</td>
<td>850.0 ± 128.42</td>
<td>114.78</td>
<td>100.23</td>
<td>180 μg/g</td>
<td></td>
</tr>
</tbody>
</table>

### Notes:
- Unless otherwise noted, values are mean ± SE (n ≥ 3).
- ND, not detectable; there was no cross-reactivity with 27.3 pmol of α-acetoxydioxifene-DNA.
- There was no inhibition with 20 μg of unmodified DNA per well, and 180 μg/well gave 50% inhibition.
strong recognition of the immunogen DNA, the antibody showed
significant recognition of TAM-DNA modified to 0.00048% or 4.8
adducts per 10^6 nucleotides (Table 1). To determine antibody
recognition of the individual adducts, we digested TAM-DNA
enzymatically, isolated adducts by HPLC, and assayed them by
DELFIA, trans and cis forms of the TAM-dG adducts showed very
low cross-reactivity, with 50% inhibition at 827 and 2229 fmol,
respectively. Even lower cross-reactivity was observed with TAM
and 4-OH-TAM, and up to 20 µg of unmodified CT DNA per well
did not give inhibition of antibody binding. When unmodified CT
DNA was subjected to every step of the immunogen preparation
procedure in the absence of α-acetoxytamoxifen, the resulting
data was not recognized by the antibody. The results indicate that
the antibody has high affinity for TAM-dG adducts when it is
present in intact DNA but not for the individual adduct, the parent
compound, or unmodified DNA.

To validate the two new immunoassays by comparison with an
established assay, we analyzed samples of TAM-DNA, modified
chemically to different levels, by DELFIA, CIA, and 32P-postlabeling.
As shown in Table 2, in seven of the nine samples, similar results
were obtained with all of the methods. However, for two samples, the
32P-postlabeling values were much lower than those determined by
CIA and/or DELFIA. At present, it is unclear whether this discrep-
ancy is due to a problem of one assay or the other, and there is
insufficient DNA to repeat them. The correlation coefficient for the
comparison between CIA and 32P-postlabeling was 0.99 and was
calculated including all nine samples. The data suggest that both CIA
and DELFIA are likely to measure TAM-DNA adducts accurately and
specifically.

Methodologies for the DELFIA and CIA are compared in Table 3.
The CIA has several advantages, including a requirement for smaller
amounts of coating antigen, higher dilution of primary and secondary
antibodies, good reproducibility for the standard curve, and a much
higher signal:noise ratio. In addition, using the TAM-DNA standard
modified to 4.8 adducts per 10^9 nucleotides, 50% inhibition occurred
at 0.92 fmol in the CIA and 20.5 fmol in the DELFIA (Fig. 1),
demonstrating that the CIA is ~20-fold more sensitive than the
DELFIA, with detection limits of 2.6 and 75 adducts per 10^9
nucleotides, respectively (Table 3).

**Experimental Studies in Rats.** For groups of four rats given a
single i.p. dose of 4.5 or 45.0 mg TAM/kg bw and euthanized 24 h
later, liver DNA was examined by TAM-DNA DELFIA. No adducts
were detected at the lower dose, but animals given the higher dose had
20.3 ± 1.7 (mean ± SE) TAM-DNA adducts per 10^9 nucleotides.
Although linearity in adduct formation would be expected, the pre-
dicted adduct level, 2 TAM-DNA adducts per 10^9 nucleotides, would
be below the detection limit for the DELFIA using 20 µg of DNA.
Because the CIA is more sensitive than the DELFIA, this assay was
used for subsequent analysis of TAM-DNA adducts in rat liver
samples.

Dose-dependent accumulation of hepatic TAM-DNA adducts in
rats given TAM for 7 days by gavage is shown in Fig. 2. Rats were
given 0, 0.5, 1.0, 5.0, 10.0, and 20.0 mg TAM/kg bw daily for 7 days
and were euthanized 24 h after the last dose. As determined by CIA,
hepatic TAM-DNA adduct levels increased with dose (Fig. 2, ○).
Similar results were obtained when the samples were analyzed by
32P-postlabeling (Fig. 2, ●), although at the highest TAM dose, CIA
appeared to underestimate the adduct level.

The persistence of TAM-DNA adducts in rat liver DNA was
determined by measuring adduct concentrations in rats given 45
mg TAM/kg bw by p.o. gavage for 4 days and euthanized at 1, 7,
10, 14, and 21 days after last TAM dose. There was a time-
dependent loss of adducts from rat liver DNA (Fig. 3), but the
values determined by CIA were lower than the values obtained by
32P-postlabeling. It is possible that some adducts determined by
32P-postlabeling, particularly those formed after chronic dosing at
high TAM concentrations, are not recognized by the anti-TAM-
DNA. Despite a discrepancy in the two assays at this high dose,
both assays demonstrated a relatively slow rate of hepatic DNA
adduct removal during a 21-day time period after 4 days of p.o.
TAM administration.

### Table 2 Comparison of 32P-postlabeling, DELFIA and CIA values (adducts per 10^9 nucleotides) for DNA modified in vitro to different levels with TAM

<table>
<thead>
<tr>
<th>TAM-DNA</th>
<th>32P-postlabeling</th>
<th>CIA</th>
<th>DELFIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3883.0</td>
<td>3622.3 ± 241.69</td>
<td>635.7 ± 21.17</td>
</tr>
<tr>
<td>2</td>
<td>417.0</td>
<td>447.7 ± 59.74</td>
<td>84.5</td>
</tr>
<tr>
<td>3</td>
<td>31.5</td>
<td>27.2 ± 3.15</td>
<td>8.7 ± 1.17</td>
</tr>
<tr>
<td>4</td>
<td>19.0</td>
<td>16.7 ± 0.48</td>
<td>6.5 ± 0.48</td>
</tr>
<tr>
<td>5</td>
<td>5.3</td>
<td>8.7 ± 0.53</td>
<td>0.6 ± 0.04</td>
</tr>
<tr>
<td>6</td>
<td>0.5</td>
<td>10.5 ± 0.68</td>
<td>17.9 ± 3.3</td>
</tr>
<tr>
<td>7</td>
<td>0.5</td>
<td>8.3 ± 1.17</td>
<td>17.9 ± 3.3</td>
</tr>
<tr>
<td>8</td>
<td>0.2</td>
<td>0.1 ± 0.04</td>
<td>0.1 ± 0.04</td>
</tr>
</tbody>
</table>

** Legend:**
- CIA: Competitive immunoassay
- DELFIA: Direct enzyme-linked fluorescent immunoassay
- NA: Not applicable

### Table 3 Comparison of CIA and DELFIA methodologies and sensitivities

<table>
<thead>
<tr>
<th>Assay conditions</th>
<th>DELFIA</th>
<th>CIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plates</td>
<td>Polystyrene clear</td>
<td>Polystyrene opaque</td>
</tr>
<tr>
<td>Precocating</td>
<td>DEAE-Dextran (18 ng)</td>
<td>DEAE-Dextran (18 ng)</td>
</tr>
<tr>
<td>Solid-phase antigen (TAM-DNA, 2.4%)</td>
<td>660 pg</td>
<td>20 pg</td>
</tr>
<tr>
<td>Blocking</td>
<td>0.05% casein</td>
<td>0.25% casein</td>
</tr>
<tr>
<td>Antiserum dilution</td>
<td>1:300,000</td>
<td>1:3,000,000</td>
</tr>
<tr>
<td>Biotinylated antirabbit IgG</td>
<td>1:2,500</td>
<td>1:5,000</td>
</tr>
<tr>
<td>Eu-labeled streptavidin</td>
<td>1:2,000</td>
<td>NA</td>
</tr>
<tr>
<td>Streptavidin-alkaline phosphatase</td>
<td>NA</td>
<td>1:6,666</td>
</tr>
<tr>
<td>1,2-Dioxetane</td>
<td>NA</td>
<td>40 mmol</td>
</tr>
<tr>
<td>Enhancement solution</td>
<td>Undiluted</td>
<td>NA</td>
</tr>
<tr>
<td>Emerald II</td>
<td>NA</td>
<td>Undiluted</td>
</tr>
<tr>
<td>Sample DNA</td>
<td>≤20 µg/well</td>
<td>≤20 µg/well</td>
</tr>
<tr>
<td>Adduct detection limit/10^9 nucleotides</td>
<td>75</td>
<td>2.6</td>
</tr>
<tr>
<td>Signal/noise ratio</td>
<td>20</td>
<td>160</td>
</tr>
</tbody>
</table>

** Legend:**
- NA: Not applicable
both the TAM-DNA CIA and $^{32}$P-postlabeling methods showed the drug from 4 to 7 days when assayed by both methods. In general, persistence of TAM-DNA adducts in rats given p.o. administration of the drug from 4 to 7 days. Data points, means for three animals; bars, SD. Inset, adduct levels at low doses (expanded).

![Graph](image)

Fig. 2. Dose-response for formation of TAM-DNA adducts in rat liver as measured by CIA (□) and $^{32}$P-postlabeling (●) in female Fischer rats given TAM at 0, 0.5, 1.0, 5.0, 10.0, and 20.0 mg TAM/kg bw by gavage daily for 7 days. Data points, means for three animals; bars, SD. Inset, adduct levels at low doses (expanded).

**DISCUSSION**

A high-titer antiserum has been elicited against TAM-DNA modified to 2.4% with α-acetoxytamoxifen. The TAM-dG adduct formed by this modification is the predominant DNA adduct found in livers of rats exposed to this drug. The antiserum has been used to develop two immunoassays, DELFIA and CIA, with a detection limit for the CIA at 2.6 adducts per $10^9$ nucleotides. By competitive DELFIA, the antiserum has been demonstrated to be specific for TAM in DNA, with cross-reactivities for the individual adduct, the drug or its metabolites, and unmodified DNA occurring only at ~1000-fold higher concentrations or not at all. The immunoassay adduct, TAM-dG, was the first TAM-DNA adduct characterized in livers of TAM-exposed rats (16). Recently, another major TAM-DNA adduct, the product of a N-desmethylated TAM metabolite, has been demonstrated in rat liver (34–37). Preliminary results indicate that TAM-DNA antiserum may recognize DNA modified with N-desmethyl-TAM-dG similarly to the immunogen DNA, in the assays described here.

The TAM-DNA CIA has been validated in a rat liver model, in which TAM-DNA adducts have been determined by both $^{32}$P-postlabeling and CIA. The data demonstrated detectable TAM-DNA adducts after a single i.p. TAM dose, and comparable dose response and persistence of TAM-DNA adducts in rats given p.o. administration of the drug from 4 to 7 days when assayed by both methods. In general, both the TAM-DNA CIA and $^{32}$P-postlabeling methods showed the same results. This was demonstrated clearly with DNA samples modified in vitro, in which no adducts would be expected to be formed from compounds other than TAM and in which the correlation coefficient between the two assays was 0.99. With DNA from rat liver, the correlation was quite good at doses lower than 20 mg TAM/kg bw, but at higher doses, the CIA gave an underestimation compared with the $^{32}$P-postlabeling, suggesting that some TAM-DNA adducts may not be recognized by the antiserum or that other adducts induced as a result of TAM exposure are being detected by $^{32}$P-postlabeling.

A large body of literature supports the associations among DNA damage, mutagenesis, and carcinogenesis. The probability that a DNA adduct can cause a mutation is related to the extent of DNA adduct formation and persistence as well as considerations of tissue specificity and mutator phenotype. Formation of DNA adducts in humans is considered an important correlate and early biomarker of cancer risk assessment. The TAM-DNA CIA, which is specific for TAM-DNA adducts and detects 2.6 adducts per $10^9$ nucleotides, may become the method of choice for determination of TAM-specific adducts in humans because the current literature in this area is associated with some controversy. Martin et al. (20) found that levels of unidentified adducts with TLC mobilities similar to TAM-related adducts were similar in livers from TAM-treated women compared with untreated women. Carmichael et al. (21, 22), in two separate studies involving a total of 38 patients, have reported no evidence of TAM-DNA adduct formation, determined by $^{32}$P-postlabeling, in endometrial biopsies from women undergoing daily p.o. TAM dosing for months to years. These investigators used $^{32}$P-postlabeling with a detection limit of 8 adducts per $10^{10}$ nucleotides (22). In addition, Phillips et al. (23) found no $^{32}$P-postlabeled DNA adducts with the chromatographic properties of known TAM-DNA adducts in leukocytes from seven women receiving TAM therapy for breast cancer. However, Hemminki et al. (24), using $^{32}$P-postlabeling followed by HPLC with detection of radiolabel, showed that leukocytes from four of six breast cancer patients receiving TAM therapy had evidence of a peak appearing at the same location as the TAM-dG standard, with a median adduct value of 5.5 adducts per $10^9$ nucleotides. The same investigators also showed an average of 2.9 adducts per $10^9$ nucleotides in endometrial DNA from five of seven women and determined the TAM-DNA adducts by $^{32}$P-postlabeling and HPLC with further chromatographic confirmation using appropriate standards (25). The interpretation of these results has been discussed in a correspondence by Orton and Topham (26). Finally, Shibutani et al. (27) have also reported finding TAM-DNA adducts in human endometrial samples using a combination of $^{32}$P-postlabeling and HPLC. The question of whether or not TAM-DNA adducts are formed in human tissues is important because it relates to issues of potential mutagenicity and tumorigenicity. However, there is evidence that TAM exposure may enhance formation of oxidative damage in DNA (38–40) and, thereby, increase the mutagenic potency of the compound beyond what might be expected from direct TAM-DNA binding. The TAM-DNA CIA, with a detection limit of 2.6 adducts per $10^9$ nucleotides, may be a useful tool to elucidate TAM genotoxicity. In addition, concentration of TAM-containing oligonucleotides by immunofluo-

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3 R. L. Divi et al., unpublished data.
ity chromatography with further analysis by an alternative method, may significantly increase TAM-DNA adduct detection capacity.

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Tamoxifen-DNA Adduct Formation in Rat Liver Determined by Immunoassay and $^{32}$P-Postlabeling

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