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Antioxidant inhibits tamoxifen–DNA adducts in endometrial explant culture

Minoti Sharma,[a,*] David E. Shubert,[a] Moheswar Sharma,[a] Kerry J. Rodabaugh,[b] Barbara P. McGarrigle,[c] Chad. M. Vezina,[c] Diane P. Bofinger,[d] and James R. Olson[c]

[a] *Department of Molecular and Cellular Biophysics, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263, USA*
[b] *Department of Gynecologic Oncology, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263, USA*
[c] *Department of Pharmacology and Toxicology, State University of New York at Buffalo, Buffalo, NY 14214, USA*
[d] *Department of Biotechnical and Clinical Laboratory Science, State University of New York at Buffalo, Buffalo, NY 14214, USA*

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Abstract

Fresh human endometrial explants were incubated for 24 h at 37 °C with either tamoxifen (10–100 μM) or the vehicle (0.1% ethanol). Three metabolites namely, α-hydroxytamoxifen, 4-hydroxytamoxifen, and *N*-desmethyltamoxifen were identified in the culture media. Tissue size was limited but DNA adducts formed by the α-hydroxytamoxifen pathway were detected using authentic α-(deoxyguanosyl-N^2) tamoxifen standards. Relative DNA-adduct levels of 2.45, 1.12, and 0.44 per 10^6 nucleotides were detected following incubations with 100, 25, and 10 μM tamoxifen, respectively. The concurrent exposure of the explants to 100 μM tamoxifen with 1 mM ascorbic acid reduced the level of α-hydroxytamoxifen substantially (68.9%). The formation of tamoxifen–DNA adducts detectable in the explants from the same specimens exposed to 100 μM tamoxifen with 1 mM ascorbic acid were also inhibited. These results support the role of oxidative biotransformation of tamoxifen in the subsequent formation of DNA adducts in this tissue.

Keywords: Tamoxifen metabolites; Tamoxifen–DNA adducts; Antioxidant; Human endometrial tissue; Explant culture

Tamoxifen (*Z*)-1-{4-[2-(dimethylamino)ethoxy]phenyl}-1,2-diphenyl-1-butene, a non-steroidal anti-estrogen used widely in the treatment of breast cancer, has also been approved for chemoprophylaxis in women at high risk of developing this disease [1,2]. While beneficial for the treatment of breast cancer, long-term tamoxifen treatment is associated with an increased risk of endometrial cancer [3,4]. Tamoxifen also has been shown to cause liver and endometrial tumors in rodents [5,6]. Studies on tamoxifen biotransformation using rodent and human liver microsomes have identified several hydroxylated derivatives [7]. Fig. 1 shows the main Phase I metabolic pathways of tamoxifen [8]. The principal sites of Phase I metabolism are the nitrogen atom of the side chain (N-oxidation and demethylation) and the 4-position (hydroxylation). Other positions also subjected to metabolism include the α-position of the ethyl side chain (hydroxylation) [9]. It was proposed that α-position is the primary site of metabolic activation as oxidative metabolism at this position was predicted to generate a resonance-stabilized carbocation capable of electrophilic attack on nucleophilic centers in DNA, leading to the formation of stable covalent DNA adducts [10]. Experimental studies have, to a large extent, borne out this hypothesis. Evidence now suggests that metabolic activation of tamoxifen to DNA binding electrophiles occurs primarily through the formation of α-hydroxytamoxifen followed by O-esterification mediated by sulfotransferase [11–14]. In rat liver, tamoxifen–DNA adducts were identified as an indication of tamoxifen genotoxicity [15,16]. Detection of tamoxifen–DNA adducts in endometrium of humans exposed to tamoxifen has been reported [17–19] although the reports are not consistent between studies [20–22].

* Corresponding author. Fax: 1-716-845-1349.
E-mail address: minoti.sharma@roswellpark.org (M. Sharma).

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The mechanism of induction of endometrial cancer by tamoxifen is not known. Studies on different replacement hormone regimens in postmenopausal women led to the conclusion that exposure to estrogen alone substantially elevates the risk of endometrial cancer [23]. It is generally accepted that metabolism of estrogen and antiestrogen occur in the liver with subsequent accumulation of metabolites in target tissue. But a potential carcinogen, 4-hydroxyestradiol, has been reported to be formed in human uterine cells from a natural estrogen, 17β-estradiol, by cytochrome P 450 (CYP) isozyme 1B1 [24,25]. Expression of CYPs in human uterine endometrium suggests that endometrial tissue has potential to generate genotoxic tamoxifen metabolites [26]. However, little is known about the ability of endometrial tissue to biotransform tamoxifen to potentially reactive tamoxifen metabolites. A previous report described the formation of α-hydroxytamoxifen in this tissue [20]. We have identified several tamoxifen metabolites in fresh human endometrial explants exposed in culture to tamoxifen [27a]. The same metabolites were also detected upon incubation of tamoxifen with recombinant human CYPs. Moreover, Western immunoblots of microsomes from human endometrium detected the presence of some of the same CYPs (2C9, 3A, 1A1, and 1B1) observed in fresh, viable endometrial explants using immunohistochemical analyses [27b]. These results support the use of explant cultures of human endometrium as a suitable in vitro model to investigate the biotransformation of tamoxifen in this target tissue. The present report describes the subsequent effects of localized biotransformation of tamoxifen on the DNA of human endometrial tissue and the potential inhibition of such effects by antioxidants using the explant culture model.

Materials and methods

Caution. Tamoxifen and its derivatives are potentially genotoxic. These compounds and human tissue used in this study were handled with proper care as advised by the Institute's Biohazard Control Office.

Materials. Tamoxifen, 4-hydroxytamoxifen, salmon testes DNA, bovine pancreas DNAase I, phosphodiesterase I from crotalus adamanteus venom, and bacterial alkaline phosphatase were purchased from Sigma–Aldrich Chemical (St. Louis, MO). HPLC grade solvents and ammonium acetate were obtained from Fisher Scientific (Bedford, MA).

Synthesis of metabolites. α-Hydroxytamoxifen and *N*-desmethyltamoxifen were synthesized following reported procedures [28,29]. α-Acetoxytamoxifen was prepared from α-hydroxytamoxifen using the method of Osborne et al. [15]. Spectroscopic characterization of the isolated products by mass spectrometry and NMR was in agreement with the literature reports.

Preparation of tamoxifen–deoxyguanosine adducts. α-Acetoxytamoxifen was reacted with salmon testes DNA using the method of Osborne et al. [15] as modified by Beland et al. [16]. The major adduct was isolated from the digested DNA by semi-preparative C18 reversed phase column (5 μm, 10 mm × 250 mm, Rainin Instrument) using a 20 min linear gradient of 10–50% acetonitrile in 0.01 M triethylammonium acetate, pH 7, at a flow rate of 4.0 ml/min. The fraction containing the adduct was collected and the solvents were removed by overnight lyophilization. Positive ion FAB-mass spectroscopy of the isolated product exhibited the parent ion at m/z 637 identifying the

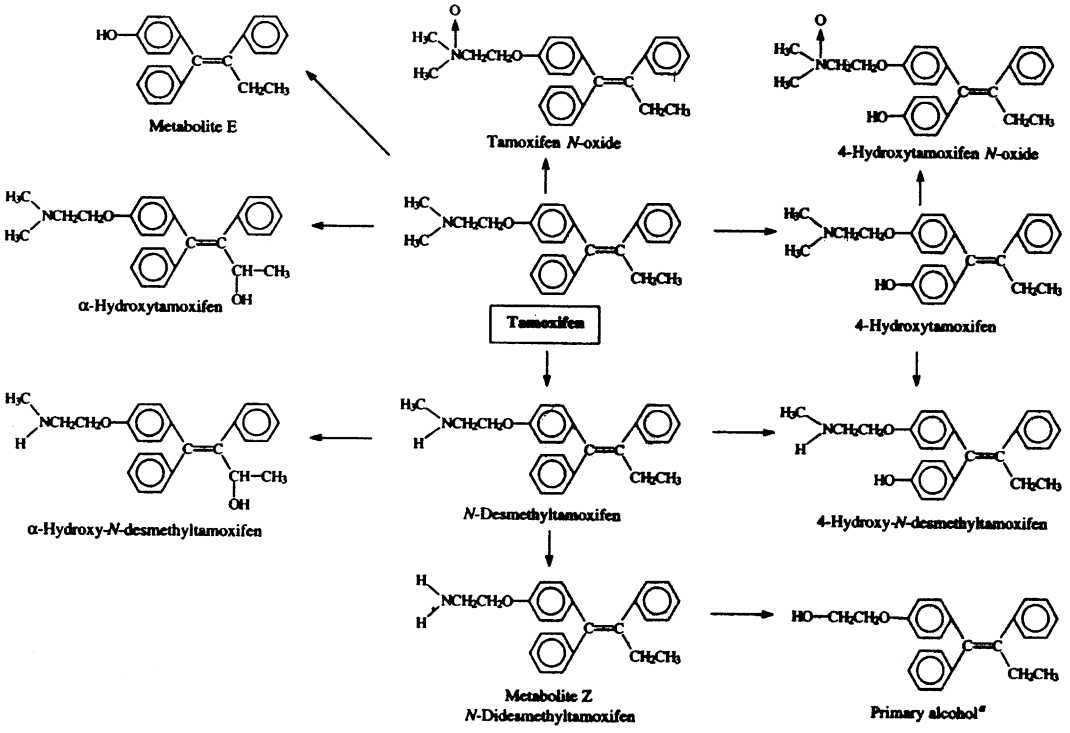

Fig. 1. Phase I metabolic pathways of tamoxifen. Adapted from [8].

molecular weight as 636 Da. The fragmentation pattern of the molecular ion at m/z 521 (loss of sugar moiety), 370 (tamoxifen moiety), 344 (bond breakage between the α-carbon and the unsaturated carbon in tamoxifen), and 178 (structural information regarding the binding site of tamoxifen in deoxyguanosine) was in agreement with the assigned (*E*)-α-(N^2-deoxyguanosinyl)tamoxifen adduct [15].

Surgical specimens. Human endometrial tissue specimens, removed at hysterectomy, were procured under IRB approved protocols from the Tissue Procurement Facility at Roswell Park Cancer Institute, with donor's consent, but without the patients' identities. Endometrial tissue was obtained from individuals, approximately 35–45 years of age and without previous history of tamoxifen exposure.

Tissue culture medium. D-MEM/F-12 medium (phenol red-free with 15 mM Hepes buffer, L-glutamine, and pyridoxine HCl; Life Technologies, Grand Island, NY) was used in all stages of tissue preparation and explant culture. D-MEM/F-12 medium was supplemented with 3% charcoal stripped fetal bovine serum (FBS; Life Technologies, Grand Island, NY), 1% antibiotic/antimycotic solution (penicillin/streptomycin/amphotericin; Life Technologies, Grand Island, NY), and 17β-estradiol (10 nM). Charcoal-stripped FBS was prepared by stirring FBS with washed and dried charcoal (30 g charcoal/1 L FBS) at 4 °C overnight, then removing the charcoal with 1.22, 0.45, and 0.22 μm filters, and performing heat inactivation at 56 °C for 30 minutes. Cultures were treated either with tamoxifen or an equivalent volume of ethanol (tamoxifen vehicle) such that the final concentration of ethanol in the medium was 0.1% and the final concentration of tamoxifen in the medium was 25 or 100 μM.

Explant culture. The surgical specimens were prepared and cultured under sterile conditions similar to the method described by Osteen et al. [30,31]. The time period between the surgical removal and explant culture was within 2 h. Typically, each sample of fresh endometrial tissue, microscopically uninvolved in disease, was placed in 3% FBS–D-MEM/F-12 medium containing 17β-estradiol (10 nM) and cut into uniform explants with a sterile scalpel blade. The pieces were immediately transferred at a concentration of 8–10 pieces per well to a 24-well plate (Costar, Cambridge, MA) containing 1 ml medium/well with 25 or 100 μM tamoxifen or vehicle (0.1% ethanol). The explants were incubated for 24 h at 37 °C in a humidified 5% CO_2–air environment. At the end of the incubation, the explants (10–50 mg/well) and culture media were harvested for HPLC analysis of adducts and metabolites, or the explants were fixed and embedded in agar for morphology and immunohistochemistry [27a,27b].

Metabolite extraction. Metabolites were extracted from culture media using 2% ethanol in hexane (5 ml/ml medium × 2) [20]. The organic extract was evaporated to dryness under reduced pressure, reconstituted with methanol (250 μl), and an aliquot (25 μl) was used for HPLC analysis.

DNA extraction and digestion. DNA was isolated from the explants using a Mannheim–Boehringer DNA isolation kit following the manufacturer's protocol. The isolated DNA was digested enzymatically to nucleosides as described earlier [32].

HPLC analysis of metabolites and DNA adducts. The metabolites were analyzed under isocratic conditions (85% methanol in 100 mM ammonium acetate, pH 5.7, flow rate 0.5 ml/min) by reversed-phase HPLC using postcolumn, online photochemical activation, and fluorescence detection [33,34]. The same technique was used to analyze the adducts except for the elution conditions. A 20 min linear gradient of 20–60% acetonitrile in 100 mM ammonium acetate, pH 5, eluted the adducts which was then followed by 60% acetonitrile isocratically for 20 min. The flow rate was 2 ml/min.

The HPLC system consisted of a binary pump system and an injection valve with variable loops (20–200 μl) from Rainin Instruments, a Radial-Pak 8MBC18 LC cartridge (10 μm, 8 mm i.d., 10 cm) with a compatible guard column from Waters, a postcolumn photochemical reactor from Aura Industries containing a 0.25 mm i.d., 5-m PTEF knitted reactor coil, and a 254-nm UV lamp which converted tamoxifen and the metabolites to fluorophores. The effluent from the photochemical converter was connected to a Shimadzu 530 RF fluorescence detector, operating at excitation and emission wavelengths of 260 and 375 nm, respectively. The detector signal was integrated by a Shimadzu Integrator CR501.

HPLC grade solvents and analytical grade reagents were used to prepare the solvent system. All solvents were filtered through a Nylon-66 filter (0.2 μm). A high pressure inline filter (SSI, 0.5 μm) was used as a further safe guard between each pump and the injector.

Results

Fig. 2 shows HPLC resolution of a mixture of authentic standards of α-hydroxytamoxifen (1), 4-hydroxytamoxifen (2), *N*-desmethyltamoxifen (3), and tamoxifen (4) using postcolumn, online photochemical activation and fluorescence detection. The elution order was based on polarity of the compounds as expected for reversed-phase chromatography. The intra- and inter-day variation in retention time and fluorescence signal (integrated peak area) were within 3%. The signal for each component was linear over a range of 1–100 ng/ml (correl. coeff. 0.99, $n = 3$). The flow velocity of the eluent through the capillary, and as a result, the residence time of the analytes in the irradiated zones appears to have some influence on the fluorescence signals (results not shown). The elution conditions were optimized to give the best resolution of the tamoxifen derivatives, not only from one another, but also from a large background of tamoxifen usually found in the tamoxifen-

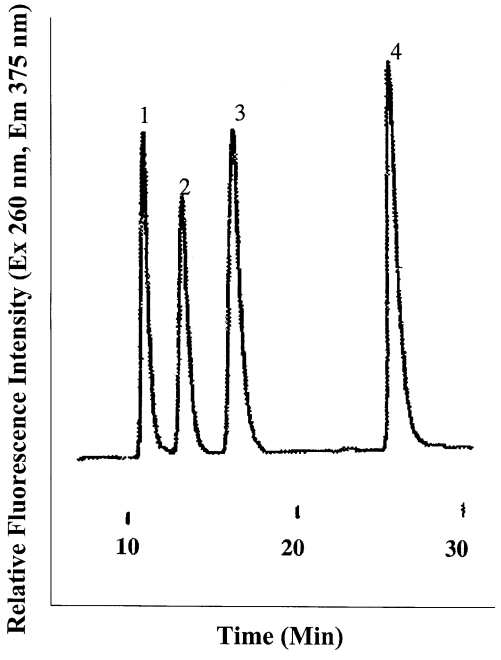

Fig. 2. Reversed-phase HPLC resolution of a mixture of tamoxifen and its three metabolites using postcolumn, online photochemical activation, and fluorescence detection; α-hydroxytamoxifen (1), 4-hydroxytamoxifen (2), *N*-desmethyltamoxifen (3), and tamoxifen (4). The elution conditions are described in Materials and methods.

exposed biological samples. Under these conditions (see methods), the lower limit of detection ($S/N = 3$) was 5, 10.0, and 2.5 pg for α-hydroxytamoxifen, 4-hydroxytamoxifen, and *N*-desmethyltamoxifen, respectively.

Fig. 3 illustrates the HPLC profiles of extracts from media of human endometrial explants exposed in culture to tamoxifen (0, 25, and 100 μM) for 24 h. Three metabolites were detected in profile (A) from 25 μM tamoxifen exposure. These metabolites were identified as α-hydroxytamoxifen (1), 4-hydroxytamoxifen (2), and *N*-desmethyltamoxifen (3) by cochromatography with authentic standards (profile B). The same metabolites were detected more readily using 100 μM tamoxifen (profile C). These peaks were not present in the extract from explants exposed to vehicle only (0.1% ethanol) (profile D).

The effect of the incubation time on the stability of the metabolites detected in Fig. 3 was further determined by incubating a mixture of the authentic metabolites of known concentration (5 ng/ml) in the culture medium at 37 °C. Aliquots were withdrawn at 0, 24, and 48 h and the metabolites were extracted and analyzed as described in Materials and methods. At each time point, the recoveries of the metabolites ranged from 92% to 96% demonstrating that the metabolites were not only stable under the incubation conditions used in this model, but the recoveries were also quantitative.

Fig. 4 illustrates the HPLC profiles of tamoxifen adduct in DNA isolated from human endometrial explants exposed in culture containing tamoxifen (10 μM) for 24 h. The peak at retention time ~19 min in profile B was identified as the major isomer of (deoxyguanosine-N^2-yl) tamoxifen adduct by cochromatography with the authentic standard (profile C). The peak detected at retention time ~20 min in profile C was not detectable in

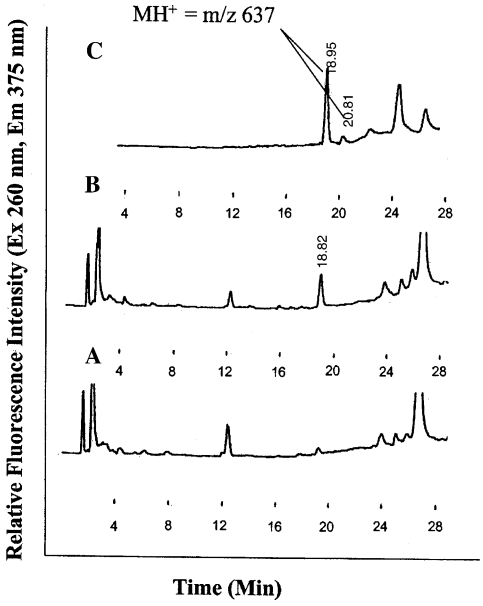

Fig. 4. HPLC analyses of tamoxifen–DNA adducts in human endometrial explants exposed in culture to (A) vehicle only, (B) 10 μM tamoxifen, and (C) cochromatography of (B) with authentic dG–tamoxifen adducts. The elution conditions are described in Materials and methods.

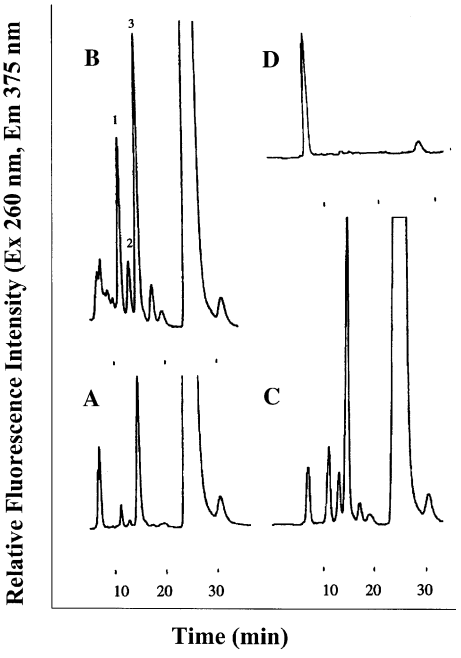

Fig. 3. HPLC analyses of metabolites in the media of human endometrial explants exposed in culture to tamoxifen; (A) 25 μM, (B) cochromatography of (A) with authentic standards, (C) 100 μM, and (D) vehicle only. Chromatographic conditions are same as in Fig. 2.

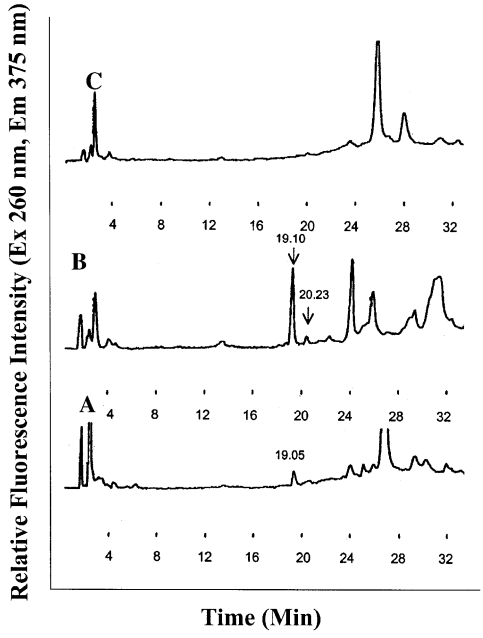

Fig. 5. HPLC analyses of tamoxifen–DNA adducts in human endometrial explants exposed in culture to (A) vehicle only, (B) 100 μM tamoxifen, and (C) 100 μM tamoxifen and 1 mM ascorbate. Chromatographic conditions are same as in Fig. 4.

Table 1
Tamoxifen biotransformation and adduct formation in human endometrial tissue exposed in culture to tamoxifen in the absence and presence of ascorbate

Dose of tamoxifen	Major tamoxifen metabolites (ng/ml) means ± SD			Tam-adduct level (adduct/10^6 nucleotides) means ± SD	
	α-OH Tam	4-OH Tam	*N*-des Tam		
10 μM	0.33 ± 0.29	0.09 ± 0.09	2.40 ± 1.47	0.44 ± 0.40	$n = 3$
25 μM	1.61 ± 1.00	0.52 ± 1.17	7.61 ± 5.08	1.12 ± 0.74	$n = 5$
100 μM	8.22 ± 8.21	6.22 ± 6.28	26.83 ± 14.36	2.45 ± 1.85	$n = 6$
100 μM Tam + 1 mM ascorbate	2.15 ± 2.29	12.07 ± 4.18	36.07 ± 14.78	ND	$n = 3$

profile B. HPLC analysis of the authentic standard always detected this minor peak along with the major peak, even after repurification of the major adduct by preparative HPLC. The molecular ion ($m/z = 637$) in positive ion FAB-mass spectrometric analysis suggests the minor peak to be either an epimer or geometric isomer of the major adduct. HPLC analysis of DNA isolated from explants exposed to vehicle only (0.1% ethanol) is shown in profile A.

Fig. 5 illustrates the HPLC profiles of tamoxifen adduct in DNA of explants exposed to higher concentration of tamoxifen (100 μM). Both the isomers of tamoxifen adducts, shown in profile Fig. 4C, were detected. This was further confirmed by cochromatography with authentic standards (results not shown). The concurrent exposure of 100 μM tamoxifen with 1 mM ascorbate inhibited the formation of tamoxifen adducts detected in the explants of the same specimen exposed to 100 μM tamoxifen alone (profile C). HPLC analysis of DNA from vehicle-exposed explants is shown in profile A.

Table 1 summarizes data from the analyses of tamoxifen metabolites and DNA adducts using the explant culture model of human endometrial tissue. Although *N*-desmethyltamoxifen was the major metabolite formed, tamoxifen–DNA adducts were identified to form through the metabolic pathway involving α-hydroxy derivative of tamoxifen using authentic standard. The table also shows the effect of antioxidant in tamoxifen metabolite formation and subsequently, in DNA modification.

Discussion

Although analysis of biotransformation of tamoxifen in extrahepatic tissue and the subsequent effects on genotoxicity are crucial for the understanding of tissue-specific action of tamoxifen, studies on localized biotransformation of tamoxifen in human are currently limited. The present study used an explant culture model, developed by Osteen et al. [30,31], that uses fresh human endometrial biopsy tissue. The advantages of this model are normal mixture of epithelial and stromal cells contained in the explants similar to that observed in vivo and the ability to assess morphology. Routine morphological evaluation is essential not only to determine tissue viability but also for assessing tissue homogeneity. The specimens utilized in this study contained >90% endometrium (results not shown). A major disadvantage of the model, however, is the small sample size (10–50 mg tissue/well) that often limits extensive time- and dose-dependent studies using drugs such as tamoxifen. For metabolism studies a 24 h incubation period with tamoxifen was chosen to optimize the CYP-mediated metabolism of tamoxifen, since CYP activities decrease with incubation time in primary culture models. Routine morphological analyses indicate that endometrial explants remain viable in specimens cultured up to 24 h in medium containing 100 μM tamoxifen [27b].

The limited sample size also demands the use of highly sensitive and yet, affordable tools for routine laboratory analyses of tamoxifen metabolites using the explant culture model of human endometrial tissue. The photochemical conversion of tamoxifen and its metabolites to phenanthrene derivatives decreased the HPLC limit of detection substantially [35,36]. Postcolumn, online UV activation using commercially available components for photoreactor (Aura Industries) in HPLC has afforded highly sensitive analysis of tamoxifen and its metabolites in human plasma and human liver microsomes with excellent reproducibility and precision [33,34]. The same technique was used in the present study for the analysis of metabolites in the culture media. As shown in Fig. 3, three metabolites were detectable, with *N*-desmethyltamoxifen representing the major metabolite. *N*-Desmethyltamoxifen was also found to be the major metabolite in endometrial samples collected during diagnostic hysteroscopy of breast cancer patients exposed to chronic tamoxifen therapy [37].

^{32}P-postlabeling technique has been the most commonly used method for DNA-adduct analysis since its development by Randerath et al. [38,39]. Our laboratory developed a novel assay for DNA damage by combining the enzymatic digestion of DNA with fluorescence postlabeling [40–43]. In the postlabeling techniques authentic modified nucleosides serve to identify the same lesions in DNA exposed to the same modifying agents. Online method that involves HPLC separation and ES-

MS/MS detection is capable of measuring DNA lesion with chemical specificity [22]. However, such a detection device is expensive and most laboratories cannot afford to use this method for routine analysis of metabolites and/or DNA adducts. We have extended the use of HPLC using postcolumn, online photochemical activation and fluorescence detection to assay tamoxifen–DNA adducts [44]. This technique does not rely on postlabeling with a fluorophore or radioisotope. We observed that by using a commercial fluorescence detector (Shimadzu RF-10 AXL), two adducts of tamoxifen were readily detectable per microgram of DNA. For assaying adducts in tamoxifen-exposed explants, vehicle-exposed controls were processed under identical conditions. The signals (integrated peak areas) generated from the corresponding controls (see profiles Figs. 4A and 5A) were subtracted from the tamoxifen-adduct signals, prior to the calculation of the relative adduct levels, shown in Table 1.

Tissue size was limited but tamoxifen adducts were detected in endometrial cultures incubated with 10, 25, and 100 μM tamoxifen (Table 1). While these results illustrate a dose-related increase in tamoxifen–DNA adducts, the variability was large between specimens. Similar variability was also noted both in the metabolites [37] and in the DNA-adduct levels detected in the endometrial samples from women exposed to tamoxifen [19]. Thus, the relevance of the explant culture model using human endometrial tissue to account for in vivo effects is well reflected. According to a recent report [22], the inconsistent results regarding the detection of tamoxifen–DNA adducts in human endometrium make the use of reliable adduct detection methodologies of critical importance. However, we and others observed that using the same methodology throughout the analyses also fails to detect tamoxifen adducts in all the samples analyzed. The data shown in Table 1 represent results from 70% of the total samples assayed. Similarly, analyses of endometrial samples in women exposed to tamoxifen also detected adducts in 50% of the total samples assayed [19]. These results suggest that interindividual differences in enzyme activities responsible not only for tamoxifen biotransformation but also in further activation of the metabolites capable of forming DNA adducts deserve further investigation to yield crucial insights regarding the tissue-specific paradoxical action of tamoxifen in human.

Dietary antioxidants have generated particular interest in defenses against cancer [45,46], but data linking antioxidant reaction to prevention of genotoxicity are limited. A role for peroxidase in the biotransformation of tamoxifen by rodent liver slices and homogenates has been reported [47]. Since human endometrial tissue is rich in peroxidase activity [48], the role of ascorbate was explored as a prototype antioxidant in tamoxifen biotransformation and its subsequent effects in adduct formation using the explant culture model of human endometrial tissue. Table 1 shows that in three independent experiments, the concurrent exposure to tamoxifen (100 μM) and antioxidant ascorbate (1 mM) inhibited the formation of detectable tamoxifen–DNA adducts in the explants from the same specimens exposed to 100 μM tamoxifen alone. Ascorbate also reduced the level of formation of α-hydroxytamoxifen substantially ($68.9 \pm 38.7\%$, $n = 3$). Ascorbate is inexpensive and remarkably nontoxic [49,50]. At 1 mM concentration ascorbate did not have impact on tissue viability. Together, these results suggest the role of oxidative biotransformation of tamoxifen in the subsequent formation of tamoxifen–DNA adducts in endometrial tissue.

Acknowledgments

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