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Peroxidase mediated dealkylation of Tamoxifen detected by ESI-MS and activation to form of DNA adducts

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Abstract

Tamoxifen (TAM) is extensively used for the treatment and prevention of breast cancer. Associated with TAM treatment is a 2- to 8-fold increase in risk of endometrial cancer. To understand the mechanisms associated with this increased risk several pathways for TAM metabolism and DNA adduct formation have been studied. The purpose of these studies has been to investigate the role of peroxidase enzymes in the metabolism of TAM and its activation to form DNA adducts. Using advanced tandem mass spectrometry we have investigated peroxidase mediated metabolism of Tamoxifen (TAM). Incubation of TAM with horseradish peroxidase (HRP) and H₂O₂, produced multiple metabolites. ESI-MS/MS analysis of the metabolites demonstrated a peak at 301.3 *m/z* with daughter ions at 183.0 *m/z*, 166.9 *m/z* 128.9 *m/z* and 120.9 *m/z* which identified the metabolite as metabolite E (ME). The levels of ME were significantly inhibited by the addition of ascorbic acid to the incubation mixture. Co-incubation of either TAM or ME and DNA with HRP and H₂O₂ produced 3 DNA adducts with a RAL $1.97 \pm 0.01 \times 10^{-7}$ and $8.45 \pm 2.7 \times 10^{-7}$. Oxidation of ME with MnO₂ produced metabolite E quinone methide (MEQM). Furthermore, incubation of either TAM or ME with HRP and H₂O₂ resulted in formation of MEQM. Reaction of calf thymus DNA with MEQM produced 3 DNA adducts with a RAL of $9.8 \pm 1.0 \times 10^{-7}$. Rechromatography analyses indicated that DNA adducts 1, 2 and 3 formed in HRP activation of either TAM or ME were the same as those formed by chemical reaction of DNA with MEQM. The results of these studies demonstrate that peroxidase enzymes can both metabolize TAM to form the primary metabolite ME and activate ME to a quinone methide intermediate, which reacts with DNA to form adducts. It is possible that peroxidase enzymes or peroxidase-like activity in endometrium could contribute to the formation of DNA damage and genotoxic effects in endometrium following TAM administration.

Keywords

Tamoxifen; Metabolite E; DNA adducts; peroxidase; HRP; quinone methide; UPLC; ESI- MS/MS

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INTRODUCTION

Tamoxifen is an antiestrogenic compound which has been demonstrated to significantly reduce the rate and time to reoccurrence of breast cancer [1–3]. More recently TAM and its derivatives are also used in prevention of breast cancer [4–6]. However associated with these beneficial effects is an increased risk for endometrial cancer in these patients [7–11]. These risks for endometrial cancer associated with TAM therapy are very similar to the increased risks for breast cancer associated with hormone replacement therapy [12,13]. In both cases, long term administration of an estrogen or estrogen like compound results in a small but significant increase risk for cancer [12–14].

The mechanism(s) by which TAM and other estrogen compounds may increase the risk for cancer in women have been extensively investigated. In rats and mice, the administration of TAM results in a dose dependent increase in the formation of DNA adducts [4,5,15,16] mutations [17–20] and liver tumor(s) [21]. The formation of TAM DNA adducts have been detected in monkeys treated with TAM [6,22] in human endometrial explants treated with TAM or its metabolites [23,24] and in women administered TAM [25–30]. In addition women administered with TAM showed an increase in K-ras mutations in endometrial tumor samples [31,32]. Although TAM DNA adducts in human samples has not been observed by all investigators [25,26] these results suggest that TAM and its metabolites are increasing the risk for endometrial cancer through genotoxic mechanisms.

TAM is extensively metabolized to 4-hydroxytamoxifen (4-HO-TAM), α -hydroxytamoxifen (α -HO-TAM), tamoxifen-N-oxide, desmethyltamoxifen, metabolite Y, metabolite E, and others in human as well as animals [33–38]. These metabolites have been shown to accumulate in endometrial tissues [39]. Our laboratory has been interested in the mechanism by which peroxidase enzymes can both metabolize compounds to form primary metabolites and also oxidize these metabolites to intermediates which react with DNA. In previous studies, we have demonstrated that two of TAM metabolites namely 4-HO-TAM and ME could be further oxidized by peroxidase enzymes to intermediates which react with DNA to form adducts. These observations with TAM metabolites have been complimented by the studies which have demonstrated that catechol estrogens, can be oxidized to the corresponding quinones and that these quinones react with DNA [40,41].

The purpose of these studies has been to investigate the peroxidase dependent conversion of TAM to primary metabolites and the further activation of these metabolites to intermediates which can react with DNA. Peroxidase enzymes are of interest because of their presence in extrahepatic tissues such as endometrium which is the site for increased cancer risk following TAM therapy [42–44]. Peroxidase enzymes have been demonstrated to carry out N-demethylation and N-oxidation reactions, and possible O-demethylation [45,46]. The formation of N-desmethyltamoxifen and tamoxifen-N-oxide have been shown in the incubation mixtures containing TAM and HRP [47]. However the O-dealkylation of TAM by peroxidase enzymes to form ME has not been studied.

The products generated by peroxidase activation of phenols and catechols are quinones and quinone methides. These quinones and quinone methides can react with DNA via Michael addition reactions to form DNA adducts [48,49]. The detection and quantitation of DNA adducts formed by the metabolites of TAM have been studied by a number of methods including ^{32}P -postlabeling [50,51] and mass spectrometry [52,53]. In addition to these methods, photoactivation of TAM to form a fluorescent phenanthrene product which provides a novel method for the detection and comparison of TAM-DNA adducts [54,60].

These current studies have demonstrated for the first time the peroxidase mediated side chain cleavage of TAM to yield ME, and that ME can be activated to MEQM which reacts

with DNA to form DNA adducts. The DNA adducts formed by peroxidase activation of TAM and its metabolites may be related to the unidentified DNA adducts observed in some of the human samples.

EXPERIMENTAL PROCEDURES

ME was prepared and purified as described [55]. TAM, calf thymus DNA, HRP, ascorbic acid and H₂O₂ were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of the highest analytical grade available.

Peroxidase metabolism of TAM

The incubation mixture consisted of 100 μ M TAM, 15 units HRP, and 100 μ M H₂O₂ in 10 mM K₂HPO₄, pH 7.4. The volumes of the incubations were 2 ml. Control assays were conducted without the addition of either H₂O₂ or HRP. Inhibitor studies were conducted with the addition of 500 μ M ascorbic acid to the assay mixture. The reactions were stopped by the addition and extraction with 1 ml of chloroform. The organic layers were concentrated, filtered and used for HPLC (not shown), MS/MS and UPLC-MS/MS analysis.

MS/MS and UPLC-MS/MS analysis of TAM metabolites

Xevo-TQ triple quadrupole mass spectrometer (Waters, Milford, MA, USA) recorded MS and MS/MS spectra using Electro Spray Ionization (ESI) in positive ion (PI) mode, capillary voltage of 3.0 kV, extractor cone voltage of 3 V and detector voltage of 500 V. Cone gas flow was set at 50 L/h and desolvation gas flow was maintained at 600 L/h. Source temperature and desolvation temperatures were set at 150 and 350 °C, respectively. The collision energy was varied between 20–29 V to optimize daughter ions. The acquisition range was 20–500 Da. Pure ME was introduced to the source at a flow rate of 10 μ l/min by using methanol: water (1:1) and 0.1% formic acid mixture as the carrier solution to optimize MS parameters and multiple reaction monitoring (MRM) method for UPLC/MS/MS operation. The organic extracts from the assay mixtures were infused at the source and MS/MS spectra were recorded.

The most abundant transition/daughter ion with the lowest accompanying background was selected for UPLC/MS/MS analysis. UPLC/MS/MS analyses of the assay samples were conducted using a Waters Acquity UPLC system connected with Xevo-TQ triple quadrupole mass spectrometer. Analytical separations on the UPLC system were conducted using an Acquity UPLC HSS T3 1.7 μ m column (1 X 150 mm) at a flow rate of 0.15 ml/min. The gradient started with 50% A (0.1% formic acid in H₂O) and 50% B (0.1% formic acid in CH₃CN), changed to 0% A over 5 min, followed by a 1-min linear gradient to 50% A, resulting in a total separation time of 6 min. The elutions from the UPLC column were introduced to the mass spectrometer and resulting data were analyzed and processed using MassLynx 4.1 software.

HRP activation of TAM or ME to form DNA adducts

The incubation mixture consisted of 100 μ M TAM or ME, 15 units HRP, 100 μ M H₂O₂ and 500 μ g of purified calf thymus DNA in 10 mM K₂HPO₄, pH 7.4. The final volumes were 2 ml. The reactions were stopped with the addition of 1 ml chloroform and extracted with chloroform: isoamylalcohol (24:1). Following extraction, the DNA was precipitated with 4 M sodium acetate and cold ethanol, and dissolved in 1 mM sodium chloride and 1.5 mM sodium acetate.

Reaction of DNA with MEQM

MEQM was prepared using MnO_2 [56,57]. MnO_2 was activated by heating at 60°C for 12 h. Eight mg of ME was dissolved in 1 ml of acetonitrile and cooled to 4°C . One hundred mg of heat activated MnO_2 was added to the solution and the reaction mixture was stirred vigorously for 10 min. After filtration, an aliquot of the product was used for recording the UV spectra or further reaction with DNA. Approximately 2 mg of MEQM was incubated with 1 mg DNA in 2.0 ml of 10 mM K_2HPO_4 , pH 7.4 for 2 h at 37°C . Following incubation, DNA was extracted and precipitated as described above.

HPLC-FL analysis of TAM adducts

From each sample, one mg of DNA was hydrolyzed to deoxyribonucleosides with a mixture of 50 units of DNase I, 4 units of snake venom phosphodiesterase and 4.5 units of alkaline phosphatase in 0.5 ml 50 mM Tris-HCL, 5 mM MgCl_2 pH 7.4 for 24 h at 37°C . Protein was precipitated by inserting the reaction mixture into a boiling water bath for 90 sec and then immediately cooling in the ice. The solution was filtered through centricon microcentrifuge tubes (Millipore, Billerica, MA) and analyzed by HPLC-FL.

HPLC-FL analysis of TAM adducts was carried out using a Perkin Elmer 265 pump coupled to a Econosphere 5μ C18 (25×4.6 mm) column (Alltech, Deerfield, IL). Post column photoactivation of the TAM derivatives was performed at 254 nm with a knitted reactor coil ($0.25 \text{ mm} \times 5 \text{ m}$) (Supelco, Bellefonte, PA) coupled to a Perkin Elmer LC 40 fluorescence detector set at 250 nm and 400 nm for excitation and emission. The mobile phase for HPLC analysis of TAM adducts consisted of 50 mM ammonium formate at a flow rate of 1 ml/min which was increased to 100% methanol over 50 min. Fluorescence response was digitized with PE 970 interface and analyzed using TurboChrom 4 software (Perkin-Elmer, Cupertino, CA, USA).

^{32}P -Postlabeling analysis of adducts

The P_1 nuclease enhanced ^{32}P -post-labeling was performed using 4 μg of DNA as previously described [50,58,59]. The radioactive mixtures were applied to 10×10 cm PEI-cellulose plates (T.J. Baker, Phillipsburg, NJ) and the DNA adducts were resolved in the following solvents: D_0 1.0 M sodium phosphate buffer (pH 6.8), D_1 2 M lithium formate and 4.8 M urea, pH 3.5, D_2 0.72 M sodium phosphate, 0.45 M Tris and 7.6 M urea, pH 8.7 and D_3 1.7 M sodium phosphate, pH 6. In the D_0 and D_1 solvent, the plates were developed from the bottom to 3–5 cm on to a paper wick. In the D_2 and D_3 solvents they were developed at a right angle to the previous direction of development to 3–5 cm on to a paper wick. The DNA adducts were located by autoradiography using Kodak XAR-5 film and a DuPont Chronex Lightning Plus intensifying screen. The areas corresponding to adduct were scraped in to scintillation vials containing 5 ml of scintillation cocktail (Safety Solve, Research Products Inc., Mount Prospect, IL) and the radioactivity was determined by scintillation counting. Areas adjacent to the radioactive spots were also scraped and counted for the determination of the background radioactivity. The relative adduct levels were calculated as previously described [50,58,59].

Rechromatography analysis of individual adducts

Individual adducts were eluted from PEI-cellulose with intermittent shaking using 0.5 ml 1.5 M triethylammonium bicarbonate (pH 7.5). The radioactivity of the eluted material was counted and aliquots containing ≈ 500 cpm activity were evaporated to dryness and redissolved in 5 μl water. These samples were applied to PEI-cellulose sheets and developed in one dimension. The solvent systems used for these rechromatographies are given in the legend to Figure 6.

RESULTS

We have investigated the metabolism of TAM by HRP/H₂O₂ using very sensitive tandem mass spectrometry. Organic extracts of assay mixtures containing TAM, HRP and H₂O₂, clearly showed peak a at 301.2 *m/z*, which after fragmentation resulted in daughter ions at 183, 166.9, 128.9, 120.9 and 104.9 *m/z* (Fig. 1A). This fragmentation pattern was matched with that of fragmentation pattern of standard ME (Fig. 1B). Further UPLC-MS/MS analysis was carried out in MRM mode to further support the formation of ME. As shown in figure 2, both synthetic ME and assay mixture showed peak (301.25 > 182.87) at *R_t* of ~4.9 min confirming the dealkylation of TAM. Other minor peaks at 3.25 and 5.15 min in the assay mixture could be either impurities or interference of other TAM metabolic products with similar ions (*m/z*) in their fragmentation pattern. Control studies were performed without the addition of either HRP or H₂O₂ to the incubation mixture. Under these conditions, formation of ME was not observed (not shown). The addition of 500 μ M ascorbic acid to the incubation inhibited the formation of ME by 98% (not shown).

It is interesting to note that the assay mixtures containing ME or TAM and HRP/ H₂O₂ showed a peak at 299.2 *m/z* indicating the presence of MEQM. Moreover, the daughter ion spectra of the peak at 299.2 *m/z* clearly matched with the fragmentation pattern of synthesized MEQM confirming the presence of MEQM (Fig. 3). UV spectral analysis of ME showed absorption maxima at 237 and 278 nm (Fig. 4A). Incubation of ME with MnO₂ resulted in shifting the absorption maxima to 358 nm. The fragmentation pattern (fig. 3A) and change in UV spectrum following oxidation by MnO₂ is consistent with ME being converted to the corresponding quinone methide MEQM in the assay mixtures. Further, MEQM was incubated with purified calf thymus DNA and the DNA adducts formed analyzed. ³²P postlabeling analysis of the DNA reacted with MEQM demonstrated the formation of two principal adducts 1 & 3 and one minor adduct 2 (Fig. 4B). The RAL produced by this reaction was $9.8 \pm 1.0 \times 10^{-7}$ and the distribution of adducts were **1**, 43.3%; **2**, 5.3% and **3**, 51.4%.

In subsequent experiments, DNA was included in the incubation mixture of TAM with HRP/H₂O₂. The formation of DNA adducts by activation of TAM with HRP/H₂O₂ was investigated using P1-enhanced ³²P-postlabeling. The results of this study demonstrated that addition of DNA to the incubation mixture containing TAM and HRP/H₂O₂ produced 3 DNA adducts. (Fig 5A). The RAL produced by this reaction was $1.19 \pm 0.91 \times 10^{-7}$ and the distribution of adducts were **1**, 63.5%; **2**, 30.1% and **3**, 6.3%. *In vitro* activation of ME with HRP and H₂O₂ also produced three DNA adducts (Fig. 5B) with a relative adduct level $8.45 \pm 2.7 \times 10^{-7}$. The distribution of the DNA adducts were **1**, 60.2%; **2**, 34.1%, and **3**, 5.5%. In incubations containing DNA, plus either TAM or ME but not HRP or H₂O₂, no DNA adducts were detected under these conditions for analysis (data not shown).

To further study the DNA adducts produced by HRP/H₂O₂ activation of TAM and ME we used a HPLC-FL method initially described by Sharma [54,60]. The advantage of this method is that it insures that a TAM derived adduct is being quantified. HPLC-FL analysis of DNA coincubated with TAM and HRP/H₂O₂ showed the presence of a single DNA adduct (**a**) with a *R_t* of 24.3 min. (Fig. 5A.) Analysis of DNA incubated with ME and HRP/ H₂O₂ also demonstrated the presence of adduct (**a**) at 24.3 min (Fig 5B).

Rechromatography experiments were performed to compare adducts formed by HRP activation of TAM and ME with the adducts formed by MEQM. The results of these comparisons demonstrated that adduct **1** formed in each of the activation systems is the same. Adduct **2** formed by HRP activation of either TAM or ME is the same and that adduct **3** formed by HRP activation of TAM is same as adduct **3** formed by MEQM. (Fig 6)

DISCUSSION

The metabolism of TAM by a peroxidase enzyme has been investigated. This study demonstrates that incubation of TAM with HRP/H₂O₂ resulted in O-dealkylation of TAM leading to the formation of ME (Fig. 1 & 2). In addition to ME, several other products, which have been previously reported were detected [47] (not shown). Furthermore, MS/MS study of the assay mixture containing TAM and HRP/ H₂O₂ clearly suggested formation of MEQM when the fragmentation patterns of peak at 299.2 *m/z* were compared to those formed in incubation mixtures of ME/HRP/ H₂O₂ and ME/MnO₂ (Fig. 3). The oxidation of ME with MnO₂ was investigated by UV and mass spectroscopy. Mixing of MnO₂ with a solution of ME resulted in a shift of the UV spectrum maxima from 237 and 278 nm to 358 nm and mass spectra of the incubation mixture clearly showed the oxidation of ME to MEQM (Fig. 3A & 4A) Addition of ascorbic acid to the incubation mixture reduced the level of ME as well as the other products by 98%. This observation suggests a radical intermediate in the formation of these products (not shown).

Incubation of DNA with MEQM resulted in the formation of 2 principal (**1** and **3**) and one minor DNA adduct (**2**). Comparison of the DNA adduct pattern produced by MEQM with that produced by ME/HRP/H₂O₂ indicates that they are similar but not identical. In both cases adduct **1** was a principal adduct. However adduct **2** was the other principal adduct by ME/HRP/H₂O₂ and adduct **3** for MEQM. The change in adduct profile of 2 and 3 produced after DNA reaction with synthetic MEQM and ME/HRP/H₂O₂ could be attributed to distinct specific activation environment (Fig. 4B & 5B). These results are consistent with ME being converted to MEQM which reacts with DNA to form the observed adducts.

Addition of DNA to the reaction mixture containing TAM and HRP/H₂O₂ resulted in the formation of DNA adducts as detected by both ³²P-postlabeling and HPLC-FL. Two principal adducts (1 and 2) and one minor adduct (3) were detected by postlabeling analysis. When the same samples were analyzed by HPLC-FL only a single DNA adduct (a) was detected. Activation of ME with HRP/H₂O₂ resulted in a DNA adduct pattern which was the same as that observed with TAM and HRP/H₂O₂, with 2 major adducts and 1 minor adduct. In addition, adduct (a) was also detected in this sample by HPLC-FL. The observed differences in number of DNA adducts observed by postlabeling and HPLC-FL suggest that under the HPLC conditions used adducts 1 and 2 are not resolved and are detected by HPLC-FL as a single adduct (a). Based on postlabeling R_f values and perceived structural similarities for adduct 1 and 2, it could be assumed that both these adducts will have similar HPLC R_f's, however there is minor possibility that one of the adduct could elute with solvent front or broad peak ~38min. These results in conjunction with those of the metabolism studies suggest that TAM was first converted to ME and then further oxidized by HRP/H₂O₂ to form MEQM (Fig 7).

Earlier it has been reported that, following its absorption, TAM is metabolized to α-HO-TAM and 4-HO-TAM by cytochrome P450 3A4 in the liver [61,62]. The results of this study demonstrate that in addition to cytochrome P450, peroxidase enzymes can also metabolize TAM to a number of primary metabolites. As demonstrated, of these primary metabolites, ME can undergo further oxidation reactions to generate reactive intermediates which bind to DNA. The results of these studies suggest that peroxidase enzymes located at extrahepatic sites could contribute to both the primary metabolism and further oxidation of TAM.

The formation of DNA adducts from the reaction of DNA with quinones and quinone methides has been established [63,65]. In these studies, the reaction of MEQM with DNA most likely occurs *via* a 1, 8 Michael addition reaction. A similar reaction has been

demonstrated to occur with the quinone methide formed by oxidation of 4-HO-TAM. The site of reaction in DNA is probably the 2 amino of deoxyguanosine (Fig 7). Isolation and identification of the DNA adducts produced by MEQM will be required to confirm this interpretation.

The mechanisms by which TAM and other estrogen compounds increase the risk for cancer are currently unknown [66,67]. One possibility is that these compounds are metabolized to intermediates which react to form DNA adducts. Although the detection of TAM-DNA adducts in endometrial samples has not been consistently observed [25–28] the formation of depurinating DNA adducts by TAM metabolites may also be possible [40,41]. The results of these studies suggest that peroxidase enzymes could contribute to this risk by metabolizing TAM to metabolites which can be further activated to form DNA adducts.

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ABBREVIATIONS

HRP	horseradish peroxidase
TAM	tamoxifen
ME	metabolite E
MEQM	metabolite E quinone methide
PEI	polyethylenimine
RAL	relative adduct level
UPLC	ultra performance liquid chromatography
ESI- MS/MS	electro-spray ionization tandem mass spectrometry

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Figure 1. MS/MS spectra of standard metabolite E (A) and metabolite E formed by peroxidase catalyzed O-dealkylation of TAM in presence of H₂O₂ (B). Plausible fragmentation of metabolite E (301.2 M+1 ion) leading to major peaks is presented.

Figure 2.
UPLC-MS/MS chromatogram of standard metabolite E (A) and metabolite E formed by peroxidase catalyzed O-dealkylation of TAM in presence of H₂O₂ (B)

Figure 3. MS/MS spectra of synthesized MEQM (A) MEQM formed by peroxidase catalyzed oxidation of metabolite E in presence of H₂O₂ (B) and MEQM formed in the assay mixture containing peroxidase, TAM and H₂O₂ (C). Plausible fragmentation of MEQM (299.2 M+1 ion) leading to major peaks is presented.

Figure 4. UV spectrum of ME (dotted line) and MEQM (solid line) prepared by oxidation of ME with MnO_2 (A). ^{32}P -postlabeling analysis of DNA adducts formed incubation of DNA with MEQM (B). The film was exposed for 8 h at -70°C .

Figure 5. Analysis of DNA adducts formed by the *in vitro* activation of TAM (A) or ME (B) with HRP and H₂O₂. The DNA adducts produced in these reactions were analyzed by HPLC-FL (Left column) and by ³²P-postlabeling (Right column). The films were exposed for 8 to 12 h at -70°C.

Figure 6.

Autoradiograms from rechromatography analysis of adducts 1, 2 and 3 formed by MnO_2 or HRP activation of ME and HRP activation of tamoxifen. Lanes a-c are adduct 1 obtained from metabolite E activated with MnO_2 (a), With HRP (b) tamoxifen and HRP (c). Lanes d & e are adduct 2 obtained from metabolite E activated with MnO_2 and DNA (d) and tamoxifen and HRP (e). Lanes f and g are adduct 3 obtained from peroxidase activation of metabolite E (f) and tamoxifen (g). Fig. 6A, lanes a-g were developed in 2.48M lithium hydroxide and 4.13M urea pH 3.35. Fig. 6B, lanes a-g were developed in 0.6M lithium chloride, 0.37M tris and 6.38M urea pH 8.0. Fig. 6C, lanes a-g were developed in 0.3M boric acid, 0.8M NaCl, 6mM EDTA and 0.8M urea pH 8.0.. The films were exposed for 50hr at -70°C

Figure 7.

Proposed model for HRP activation of TAM to form ME and DNA adducts *in vitro*. This model illustrates how TAM undergoes O-dealkylation to give ME, which is further activated by HRP to form the quinone methide MEQM. In this model MEQM undergoes a 1,8 Michael addition reaction with deoxyguanosine to form ME-adduct.