4-Hydroxylated Metabolites of the Antiestrogens Tamoxifen and Toremifene Are Metabolized to Unusually Stable Quinone Methides

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Received August 3, 1999

Tamoxifen is widely prescribed for the treatment of hormone-dependent breast cancer, and it has recently been approved by the Food and Drug Administration for the chemoprevention of this disease. However, long-term usage of tamoxifen has been linked to increased risk of developing endometrial cancer in women. One of the suggested pathways leading to the potential toxicity of tamoxifen involves its oxidative metabolism to 4-hydroxytamoxifen, which may be further oxidized to an electrophilic quinone methide. The resulting quinone methide has the potential to alkylate DNA and may initiate the carcinogenic process. To further probe the chemical reactivity and toxicity of such an electrophilic species, we have prepared the 4-hydroxytamoxifen quinone methide chemically and enzymatically, examined its reactivity under physiological conditions, and quantified its reactivity with GSH. Interestingly, this quinone methide is unusually stable; its half-life under physiological conditions is approximately 3 h, and its half-life in the presence of GSH is approximately 4 min. The reaction between 4-hydroxytamoxifen quinone methide and GSH appears to be a reversible process because the quinone methide GSH conjugates slowly decompose over time, regenerating the quinone methide as indicated by LC/MS/MS data. The tamoxifen GSH conjugates were detected in microsomal incubations with 4-hydroxytamoxifen; however, none were observed in breast cancer cell lines (MCF-7) perhaps because very little quinone methides is formed. Toremifene, which is a chlorinated analogue of tamoxifen, undergoes similar oxidative metabolism to give 4-hydroxytoremifene, which is further oxidized to the corresponding quinone methide. The toremifene quinone methide has a half-life of approximately 1 h under physiological conditions, and its rate of reaction in the presence of excess GSH is approximately 6 min. More detailed analyses have indicated that the 4-hydroxytoremifene quinone methide reacts with two molecules of GSH and loses chlorine to give the corresponding di-GSH conjugates. The reaction mechanism likely involves an episulfonium ion intermediate which may contribute to the potential cytotoxic effects of toremifene. Similar to what was observed with 4-hydroxytamoxifen, 4-hydroxytoremifene was metabolized to di-GSH conjugates in microsomal incubations at about 3 times the rate of 4-hydroxytamoxifen, although no conjugates were detected with MCF-7 cells. Finally, these data suggest that quinone methide formation may not make a significant contribution to the cytotoxic and genotoxic effects of tamoxifen and toremifene.

Introduction

With recent approval of tamoxifen (Nolvadex) for the prevention of breast cancer by the Food and Drug Administration, a deeper understanding of the risk factors involved with long-term usage of this drug is crucial. Despite the beneficial effects of tamoxifen for women who are at risk of developing breast cancer, reports have shown that tamoxifen may increase the incidence of endometrial cancer in women (*1*−*7*), may induce the formation of DNA adducts (*8*−*10*), and causes hepatocarcinoma in animal models (*11*−*14*). Several bioactivation pathways leading to the toxicity of tamoxifen have been proposed. They include the generation of reactive intermediates such as the tamoxifen carbocation (*15*−*17*), *o*-quinone (*18*, *19*), and quinone methide (*20*−*22*). The mechanism of formation of the quinone methide likely involves P450-catalyzed aromatic hydroxylation of tamoxifen generating 4-hydroxytamoxifen, which undergoes two-electron oxidation of the π system resulting in the quinone methide (Scheme 1) (*23*−*26*).

Recently, it has been shown that the 4-hydroxytamoxifen quinone methide reacts with DNA to form covalent adducts in vitro (*22*). However, very little is known about the reactivity, rate of formation, and other potential biological targets of this electrophile. To further probe the chemical reactivity and toxicity of the tamoxifen quinone methide, we have synthesized it chemically and enzymatically, measured its half-life in solution, quantified its reactivity with GSH,[1] and characterized the GSH conjugates. Interestingly, we have found that very little quinone methide was formed in microsomal incubations with 4-hydroxytamoxifen. In addition, the reaction between this quinone methide and GSH may be a reversible

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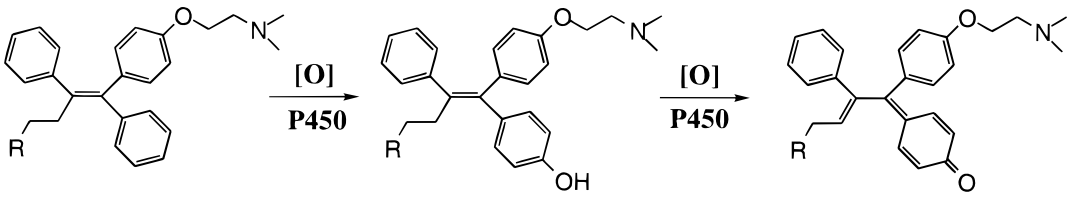
10.1021/tx990144v CCC: $19.00
Published on Web 12/18/1999

Scheme 1. Biotransformation of Tamoxifen and Toremifene to the Corresponding Quinone Methides



R = H = Tamoxifen
R = Cl = Toremifene

process because its GSH conjugates slowly decompose over time, regenerating the quinone methide. As a result, quinone methide formation from tamoxifen may not play a major role in the carcinogenic effects attributed to the parent compound.

Toremifene is a chlorinated structural analogue of tamoxifen. It was introduced in the late 1980s for the treatment of advanced stages of breast cancer (*27*). 4-Hydroxytoremifene, which may play a role similar to that of 4-hydroxytamoxifen (*28*), is one of the metabolites of toremifene (*29*) which could be further oxidized to an electrophilic quinone methide. Therefore, we have investigated the rate of formation of the 4-hydroxytoremifene quinone methide in microsomal incubations, measured its half-life under physiological conditions, and examined the conjugates formed with GSH. Unlike the tamoxifen quinone methide, the toremifene quinone methide forms di-GSH conjugates through a mechanism which likely involves an additional electrophilic intermediate, the episulfonium ion. It is possible that episulfonium ion formation contributes to the biological effects of toremifene.

Materials and Methods

Caution: *The quinone methides were handled in accordance with NIH guidelines for the Laboratory Use of Chemical Carcinogens (30).* All chemicals were purchased from Aldrich (Milwaukee, WI), Fisher Scientific (Itasca, IL), or Sigma (St. Louis, MO) unless stated otherwise. [^{3}H]GSH (glycine-2-^{3}H) was obtained from Dupont (Boston, MA) and diluted to a specific activity of 40 nCi/nmol. The biologically active isomers (*Z*)-4-hydroxytamoxifen and (*Z*)-4-hydroxytoremifene were stereoselectively synthesized by the McMurry reaction as described previously (*31*).

Instrumentation. HPLC experiments were performed on a Shimadzu LC-10A gradient HPLC system equipped with an SIL-10A autoinjector, an SPD-M10AV UV/vis photodiode array detector, and an SPD-10AV detector. Peaks were integrated with Shimadzu EZ-Chrom software and a 486 personal computer. UV spectra were measured on a Hewlett-Packard 8452A photodiode array UV/vis spectrophotometer. ^{1}H NMR spectra were obtained with a Bruker Avance DPX300 spectrometer at 300 MHz. Positive ion electrospray mass spectra were obtained using a Hewlett-Packard 5989B engine quadrupole mass spectrometer equipped with a ChemStation data system and high-flow pneumatic nebulizer-assisted electrospray LC/MS interface. The mass spectrometer was interfaced with a Hewlett-Packard (Palo Alto, CA) 1050L gradient HPLC system equipped with a photodiode array UV/vis absorbance detector set at 230−450 nm. The temperature of the quadrupole analyzer was maintained at 120 °C, and unit resolution was used for all measurements. Nitrogen at a pressure of 80 psi was used for nebulization of the HPLC effluent, and a nitrogen bath gas at 300 °C and a flow rate of 10 L/min were used for evaporation of the solvent from the electrospray. A mass range from 300 to 1050 mass units was scanned every 1.5 s. Tandem MS (MS/MS) spectra were obtained using a Micromass (Manchester, U.K.) Quattro II triple-quadrupole mass spectrometer equipped with an electrospray ionization source and a Hewlett-Packard 1050 HPLC system. Collision-induced dissociation (CID) was carried out using a range of collision energy from 25 to 70 eV and argon collision gas pressure of 2.7 μbar.

HPLC Methodology. Two general methods were used to analyze and separate the quinone methides and the GSH conjugates. All retention times reported in the text were obtained using method A.

Method A. Analytical HPLC analysis was performed using a 4.6 mm × 150 mm Ultrasphere C-18 column (Beckman) on the above-mentioned Shimadzu HPLC system. The mobile phase consisted of 20% methanol in 0.25% perchloric acid/0.25% acetic acid (pH 3.5) with a flow rate of 1.0 mL/min for 5 min, which was increased to 35% CH_3OH over the course of 1 min, then to 60% over the course of the next 39 min, and finally to 95% CH_3OH over the course of the last 5 min.

Method B. LC/MS analysis was performed using a 4.6 mm × 150 mm Ultrasphere C-18 column (Beckman) on the above-mentioned Hewlett-Packard LC/MS system, and tandem mass spectra were obtained using the Micromass system interfaced with the Hewlett-Packard HPLC system. The mobile phase consisted of 20% methanol in 0.5% ammonium acetate (pH 3.5) with a flow rate of 1.0 mL/min for 5 min, which was increased to 35% CH_3OH over the course of 1 min, then to 60% over the course of the next 39 min, and finally to 95% CH_3OH over the course of the last 5 min.

Preparation of the Tamoxifen and Toremifene Quinone Methides. Typically, (*Z*)-4-hydroxytamoxifen or (*Z*)-4-hydroxytoremifene (2 mg) was dissolved in acetone (1 mL) in the dark and cooled to 0−5 °C. Upon careful addition of freshly prepared manganese dioxide [20 mg (*22*)], the suspension was stirred for 1 h and centrifuged. The yellow supernatant containing 4-hydroxytamoxifen quinone methide or 4-hydroxytoremifene quinone methide was removed and concentrated to a final volume of 500 μL under N_2. An aliquot (25 μL) of the supernatant was immediately analyzed by LC/MS (method B). **4-Hydroxytamoxifen quinone methide:** UV (CH_3CN) 200, 230, 280, 400 nm; positive ion electrospray MS *m/z* (rel intensity) 386 (100%) $[M + H]^+$; MS/MS of *m/z* 386 (rel intensity) 341 (20%), 314 (10%), 221 (13%), 128 (5%), and 72 (100%); retention time of 52 min. 4-Hydroxytoremifene quinone methide was prepared similarly from (*Z*)-4-hydroxytoremifene and analyzed in a similar manner. **4-Hydroxytoremifene quinone methide:** UV (CH_3CN) 208, 238, 302, 382 nm; positive ion electrospray MS *m/z* (rel intensity) 420 (100%) $[M + H]^+$.

Kinetic Experiments. The disappearance of either 4-hydroxytamoxifen quinone methide or 4-hydroxytoremifene quinone methide (0.16 mM) in 50 mM K_2HPO_4 buffer (10 mL, pH 7.4, 37 °C) was followed by monitoring the decrease in UV absorbance at 400 nm (5 min/scan) using a Hewlett-Packard 8452A diode array spectrophotometer. Initially, an aliquot (1 mL) of the reaction solution was removed, and its absorbance was scanned. Aliquots (1 mL) were then removed every 4 min and centrifuged at 13 000 rpm for 30 s to remove any precipitate

[1] Abbreviations: 4-OHTAMQM, 4-hydroxytamoxifen quinone methide; 4-OHTORQM, 4-hydroxytoremifene quinone methide; GSH, glutathione; 4-OHTAM−SG, glutathione conjugates of 4-hydroxytamoxifen; 3,4-di-OHTAM−SG, glutathione conjugates of 3,4-dihydroxytamoxifen; 4-OHTOR−diSG, disubstituted glutathione conjugates of 4-hydroxytoremifene.

Table 1. Reactivity of the Tamoxifen and Toremifene Quinone Methides[a]

substrate	phosphate buffer half-life (min)	50 mM GSH half-life (min)
4-OHTAMQM	174 ± 41	3.9 ± 0.1
4-OHTORQM	57 ± 8	5.7 ± 0.3

[a] The rates of disappearance of quinone methides at 410 nm as described in Materials and Methods (pH 7.4, 37 °C). Results are the means ± SD of three determinations.

before recording the absorbance. Finally, an aliquot of the quinone methide solution in buffer was left in the quartz cuvette, and its rate of decomposition at 410 nm was continuously monitored at the same scan rate. Rate constants were determined in triplicate for at least six half-lives (Table 1). The disappearance of either 4-hydroxytamoxifen quinone methide (0.16 mM) or 4-hydroxytoremifene quinone methide (0.14 mM) in the presence of GSH (50 mM) in phosphate buffer (50 mM, pH 7.4) was followed by monitoring the decrease in absorbance at 400 nm (10 s/scan) at 37 °C. Rate constants were determined as described above (Table 1).

GSH Conjugates of the Tamoxifen and Toremifene Quinone Methides. GSH conjugates of 4-hydroxytamoxifen quinone methide were prepared by incubating the quinone methide (1.6 mM) with 50 mM GSH in 50 mL of K_2HPO_4 buffer (50 mM, pH 7.4) at 37 °C for 30 min. The reaction was terminated by extracting the aqueous solution with 50 mL of ether. The GSH conjugates were isolated from the aqueous phase on PrepSep C_{18} extraction cartridges (Fisher Scientific, Fair Lawn, NJ) and eluted with methanol. The methanol was concentrated to a final volume of 200 μL, and an aliquot (25 μL) was analyzed using LC/MS (method B). **4-OHTAM−SG 1:** UV (CH_3OH) 245, 275 nm; positive ion electrospray MS *m/z* (rel intensity) 693 (100%) $[M + H]^+$; retention time of 25 min. **4-OHTAM−SG 2 and 3 (unresolved):** UV (CH_3OH) 246, 275 nm; positive ion electrospray MS *m/z* (rel intensity) 693 (100%) $[M + H]^+$; retention time of 28 min. **4-OHTAM−SG 4:** UV (CH_3OH) 246, 275 nm; ^{1}H NMR (CD_3OD) δ 1.18 (t, 3H, C**H**$_3$), 1.88 (m, 2H, Gluβ), 2.45 (m, 2H, Gluγ), 2.88 (m, 1H, Cysβ), 2.94 [s, 6H, N(C**H**$_3$)$_2$], 3.29 (m, 1H, Cysβ), 3.59 (t, 2H, C**H**$_2$N), 3.59 (m, 1H, Gluα), 3.76 (d, 2H, Glyα), 4.33 (m, 1H, Cysα), 4.60 (m, 1H, CH$_3$**H**) 4.68 (t, 2H, OC**H**$_2$), 6.65 (d, 2H, J = 5.2 Hz, OPh**H**), 6.99 (d, 2H, J = 5.2 Hz, OPh**H**), 7.07 (d, 2H, J = 5.2 Hz, OPh**H**), 7.23−7.66 (7H, m, Ar**H**); positive ion electrospray MS *m/z* (rel intensity) 693 (100%) $[M + H]^+$; MS/MS of *m/z* 693 (rel intensity) *m/z* 564 (30%) $[MH - Glu]^+$, 386 (100%) $[MH - GSH]^+$, 341 (10%) $[MH - GSH - HN(CH_3)_2]^+$; retention time of 30 min. A similar procedure was used to generate the 4-hydroxytoremifene quinone methide GSH conjugates. **4-OHTOR-diSG 1 and 2 (partially resolved):** UV (CH_3OH) 250, 280 nm; positive ion electrospray MS *m/z* (rel intensity) 998 (15%) $[M + H]^+$, 500 (100%) $[M + 2H]^{2+}$; MS/MS of *m/z* 998 (major ions) (rel intensity) *m/z* 869 (10%) $[MH - Glu]^+$, 691 (95%) $[MH - GSH]^+$, 673 (30%) $[MH - GSH - H_2O]^+$, 562 (60%) $[MH - GSH - Glu]^+$; retention time of 13 min. **4-OHTOR-diSG 3 and 4 (partially resolved):** UV (CH_3OH) 245, 280 nm; positive ion electrospray MS *m/z* (rel intensity) 998 (15%) $[M + H]^+$, 500 (100%) $[M + 2H]^{2+}$; MS/MS of *m/z* 998 (rel intensity) *m/z* 869 (10%) $[MH - Glu]^+$, 850 (12%) $[MH - Glu - H_2O]^+$, 691 (30%) $[MH - GSH]^+$, 673 (20%) $[MH - GSH - H_2O]^+$, 562 (50%) $[MH - GSH - Glu]^+$; retention time of 16 min.

Incubations. Female Sprague-Dawley rats (180−200 g) were obtained from Sasco Inc. (Omaha, NE). The rats were pretreated with dexamethasone to induce P450 3A isozymes. They were given dexamethasone in corn oil (100 mg/kg) daily via ip injection for 3 days and sacrificed on day 4. Protein and P450 concentrations of the liver microsomes were determined as described by Thompson et al. (*32*). Incubations containing microsomal protein (1 nmol of P450/mL) were conducted for 15 min at 37 °C in 50 mM phosphate buffer (pH 7.4, 0.5 mL total volume). Substrates (4-hydroxytamoxifen or 4-hydroxytoremifene) were added as solutions in DMSO, and [^{3}H]GSH (specific activity of 40 nCi/nmol) was added in phosphate buffer, to achieve final concentrations of 0.5 and 2.5 mM, respectively. An NADPH-generating system consisting of 1 mM $NADP^+$, 5 mM isocitric acid, and 0.2 unit/mL isocitric acid dehydrogenase was used together with 5.0 mM $MgCl_2$. For control incubations, $NADP^+$ was omitted. The reactions were initiated by the addition of $NADP^+$ and terminated by chilling in an ice bath followed by the addition of perchloric acid (25 μL).

Adduct Quantification. The incubates were centrifuged at 13 000 rpm for 5 min to precipitate microsomal protein. Aliquots of the supernatant (100 μL) were analyzed directly by HPLC (method A). For quantification of GSH conjugates, aliquots (300 μL) of the column effluent were collected during each run, and radioactivity was measured with a Beckman model LS 5801 liquid scintillation counter. Concentrations of the GSH conjugates were calculated by adding the radioactivities associated with each peak and converting the data to nanomolar amounts using the specific activity of [^{3}H]GSH (Table 2).

Direct Detection of the Tamoxifen Quinone Methide in Microsomal Incubations. In another set of experiments, 4-hydroxytamoxifen was incubated with rat liver microsomes using the same incubation conditions described above except GSH was omitted and the total incubation volume was 10 mL. A different workup procedure was adopted to preserve 4-hydroxytamoxifen quinone methide generated from microsomal incubation. The incubates were saturated with NaCl and extracted with ethyl acetate (2 × 10 mL). The combined ethyl acetate layers were concentrated to 500 μL under nitrogen and diluted with 1 mL of acetonitrile. An aliquot (25 μL) was then analyzed using LC/MS (method B).

Incubation of 4-Hydroxytamoxifen and 4-Hydroxytoremifene in MCF-7 Cells. Briefly, MCF-7 cells were maintained in MEME supplemented with 1% penicillin, 10 mg/L streptomycin, 10 mg/L fungizome, 1% nonessential amino acids, and 10% bovine serum. The cells were harvested by trypsinization, counted, and diluted to a density of ~10^6 cells/mL of medium. Aliquots (10 mL) of the diluted cells were transferred to three pasteurized incubation tubes. 4-Hydroxytamoxifen (or 4-hydroxytoremifene) was added to yield final concentrations of 0, 20, and 50 μM. DMSO was used as the control. The cells were then incubated at 37 °C for 30 min. Incubations were terminated by chilling the tubes containing the cells in an ice bath followed by addition of perchloric acid (50 μL/mL) to each tube. The incubates were centrifuged at 13 000 rpm for 6 min to precipitate cellular debris. After centrifugation, the supernatants were extracted using C_{18} solid-phase extraction cartridges (Oasis; HLB Cartridge, Waters Corp., Milford, MA) and eluted with methanol. The methanol was concentrated to a final volume of 300 μL, and aliquots (150 μL) were analyzed directly by HPLC using method A.

Results and Discussion

Chemical Generation of the Tamoxifen and Toremifene Quinone Methides. Mild oxidants such as silver(I) oxide (*22*, *33*), lead(IV) oxide (*34*, *35*), and manganese(IV) oxide (*36*) have been reported for the efficient synthesis of quinones and quinone methides from various phenolic compounds. In this study, we found that activated γ-manganese dioxide served as the most effective oxidant for 4-hydroxytamoxifen (*22*). The UV spectra exhibited a strong absorbance around 400 nm, which was absent in the starting material and was characteristic of most quinones and quinone methides (*34*, *35*). LC/MS analysis revealed the appearance of a new peak with a retention time of 52 min and a protonated molecular ion at *m/z* 386. MS/MS analysis of *m/z* 386 with CID gave the fragment ion spectra shown in Figure 1, which strongly suggested that the new peak was 4-hydroxytamoxifen quinone methide. Similar oxidation experiments with 4-hydroxytoremifene gave the

Table 2. Conversion of 4-Hydroxytamoxifen and 4-Hydroxytoremifene to the GSH Conjugates by Rat Liver Microsomes[a]

substrate	conjugate	retention time (min)	rate of formation [nmol (nmol of P450)$^{-1}$ (10 min)$^{-1}$]
4-hydroxytamoxifen quinone methide	4-OHTAM−SG 1	25	0.0064 ± 0.0008
	4-OHTAM−SG 2 and 3	28	0.011 ± 0.003
	4-OHTAM−SG 4	30	0.0058 ± 0.0014
			total: 0.023 ± 0.0052
o-quinone	3,4-di-OHTAM−SG 1	34	0.0069 ± 0.002
	3,4-di-OHTAM−SG 2	37	0.0047 ± 0.0002
	3,4-di-OHTAM−SG 3	41	0.0037 ± 0.0002
			total: 0.015 ± 0.0024
4-hydroxytoremifene quinone methide	4-OHTOR−diSG 1 and 2	13	0.06 ± 0.018
	4-OHTOR−diSG 3 and 4	16	0.031 ± 0.006
			total: 0.091 ± 0.024

[a] Incubations were conducted for 15 min with 0.5 mM substrate, 2.5 mM [^{3}H]GSH (specific activity of 40 nCi/nmol), liver microsomes from dexamethasone-induced female rats (1 nmol of P450/mL), and an NADPH-generating system. Results are the means ± SD of three determinations.

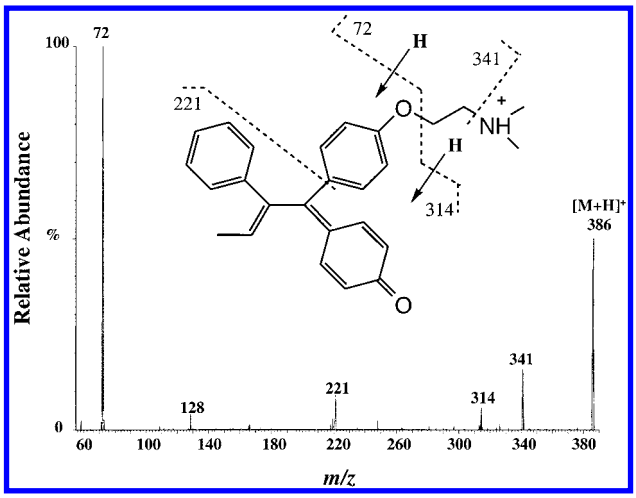


Figure 1. MS/MS fragmentation pattern of 4-hydroxytamoxifen quinone methide.

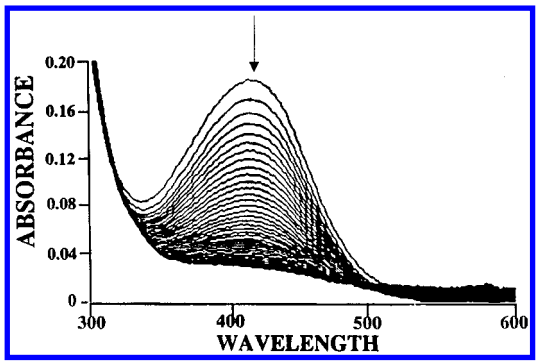
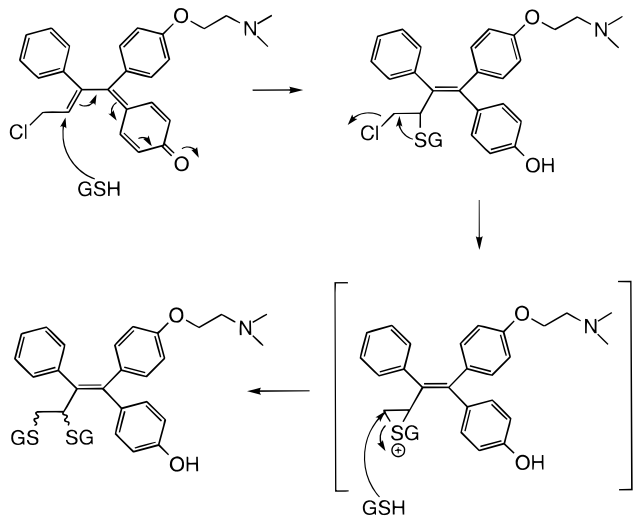


Figure 2. Kinetic studies on the reaction of 4-hydroxytamoxifen quinone methide with GSH. Incubations contained 4-hydroxytamoxifen (0.16 mM) and GSH (50 mM) at pH 7.4 and 37 °C. UV scans were over the 200−600 nm wavelength range, at a rate of 10 s/scan.

Scheme 2. Reaction of 4-Hydroxytoremifene Quinone Methide with GSH with an Initial 1,8-Michael Addition of One Molecule of GSH to the Quinone Methide Followed by the Formation of an Episulfonium Ion Which Reacts with a Second GSH Molecule To Give a Disubstituted GSH Conjugate



expected protonated molecule at *m*/*z* 420 in the positive ion electrospray mass spectrum. Furthermore, the solution containing 4-hydroxytoremifene quinone methide exhibited a strong UV absorbance around 400 nm as expected for quinone methide generation.

Reactivity of the Tamoxifen and Toremifene Quinone Methides. There have been some reports in the literature about the chemistry and reactivity of *p*-quinone methides (*37*, *38*). They are known for their electrophilicity and low capacity for redox chemistry (*39*). To explore the reactivity of tamoxifen or toremifene quinone methides, we measured their half-lives under physiological conditions and in the presence of excess GSH. Interestingly, the quinone methide derived from 4-hydroxytamoxifen is unusually stable; its half-life under physiological conditions was approximately 3 h, and its half-life in the presence of excess GSH was approximately 4 min (Figure 2 and Table 1). The unusual stability is readily explained by the contribution of an extensive resonance stabilization (conjugated π system) to the overall resonance hybrid of the quinone methide. The 4-hydroxytoremifene quinone methide had a half-life of approximately 1 h under physiological conditions, and its rate of reaction in the presence of excess GSH was approximately 6 min (Table 1). More detailed analysis indicated that the 4-hydroxytoremifene quinone methide reacts with two molecules of GSH and loses chlorine to give the corresponding di-GSH conjugates as described below. This substitution reaction may be facilitated by a three-membered ring sulfonium ion intermediate (episulfonium ion, Scheme 2). A GSH molecule first reacts with 4-hydroxytoremifene quinone methide via 1,8-Michael addition to generate a half-mustard intermediate (*40*). The thiol group then displaces the chlorine and forms an episulfonium ion. Finally, a

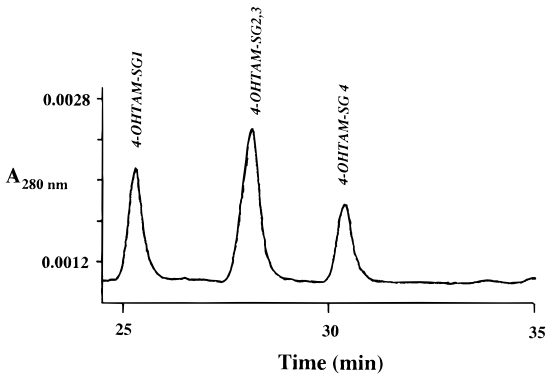
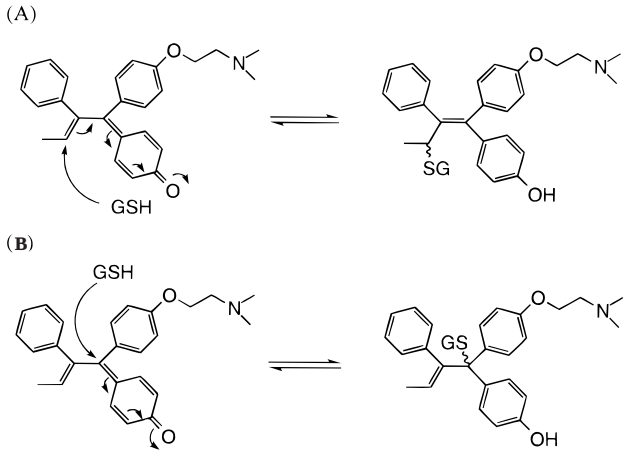


Figure 3. HPLC chromatogram of 4-hydroxytamoxifen−GSH conjugates. 4-OHTAM−SG 2 and 3 were resolved using a different HPLC method (unpublished results).

second GSH molecule reacts with the episulfonium ion to give the disubstituted GSH conjugates (Scheme 2). There have been several reports about the possible involvement of an episulfonium ion as a reaction intermediate in the reactions of GSH with various known chemical carcinogens (*40*−*46*). As a result, it is possible that the episulfonium ion contributes to the potential cytotoxic effects of toremifene.

GSH Conjugates of 4-Hydroxytamoxifen Quinone Methide. Glutathione has been shown to be an effective agent for trapping quinone methides due to the nucleophilicity of the cysteine sulfhydryl group and the relatively high concentration of GSH in vivo. We have found that incubation of the 4-hydroxytamoxifen quinone methide with GSH resulted in the disappearance of the distinct yellow color, indicating that a reaction had taken place. An HPLC method for separating and characterizing the GSH conjugates by UV and electrospray MS was developed. Four GSH-dependent peaks (4-OHTAM−SG 1−4, Figure 3) were detected in the HPLC chromatogram, two of which were unresolved (peaks 2 and 3). On the basis of the qualitative comparison between the total peak area of GSH conjugates formed from the quinone methide and the total peak area of unreacted 4-hydroxytamoxifen, the chemical oxidation process was only 17% efficient. Four GSH-dependent peaks were expected since GSH could react with 4-hydroxytamoxifen quinone methide either through 1,8-Michael addition to give two isomers of 4-OHTAM−SG adducts (*E* and *Z* isomers) or through 1,6-Michael addition to give two additional isomers (Scheme 3; *35*). We have found that these GSH adducts are unstable and decompose slowly over time (7 days). As a result, we were only able to partially purify one of the GSH conjugates (4-OHTAM−SG 4). The ^{1}H NMR chemical shifts obtained for this conjugate are given in Materials and Methods. The assignments were based on a comparison of ^{1}H NMR data for 4-hydroxytamoxifen and other tamoxifen−GSH adducts published in ref *54*. However, it was difficult to make specific assignments for all the peaks because of solvent peak interference. Nevertheless, the position of the GSH molecule is likely to be at the α position. The product ion spectra of the protonated molecule of 4-OHTAM−SG 4 (*m*/*z* 693) gave abundant fragment ions at *m*/*z* 564 (30% rel intensity) $[MH - Glu]^+$, 386 (100% rel intensity) $[MH - GSH]^+$, and 341 (10% rel intensity) $[MH - GSH - HN(CH_3)_2]^+$. Loss of 129 mass units (pyroglutamic acid) from the protonated molecule generated the fragment ion at *m*/*z* 564, which is indicative of a typical GSH conjugate

Scheme 3. Reaction of 4-Hydroxytamoxifen Quinone Methide with GSH[a]



[a] (A) 1,8-Michael addition of GSH to the quinone methide and (B) 1,6-Michael addition of GSH to the quinone methide.

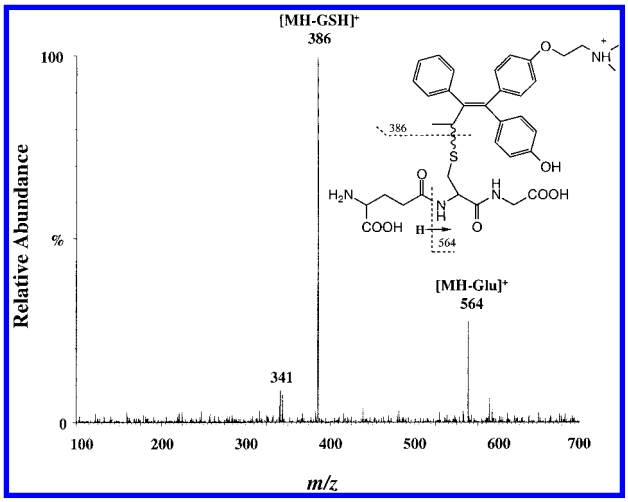


Figure 4. MS/MS fragmentation pattern of the 4-hydroxytamoxifen−GSH conjugate.

fragmentation pattern in MS/MS (Figure 4) (*47*−*49*). The fragment ion at *m*/*z* 341 is generated from the fragment ion at *m*/*z* 386 after losing the dimethyl amino group. After ^{1}H NMR data had been obtained, LC/MS analysis of this conjugate showed the appearance of a new peak (retention time of 52 min) at *m*/*z* 386, suggesting that the quinone methide had been regenerated. Tandem mass spectrometry of the *m*/*z* 386 ion gave the same fragment ion spectra as the standard 4-hydroxytamoxifen quinone methide (Figure 1), strongly suggesting that the new peak is the quinone methide. This also suggests that the reaction between 4-hydroxytamoxifen and GSH is a reversible process.

GSH Conjugates of 4-Hydroxytoremifene Quinone Methide. In contrast with the tamoxifen quinone methide, the reaction between 4-hydroxytoremifene quinone methide and GSH resulted in the formation of di-GSH conjugates. Four GSH-dependent peaks were detected in the HPLC chromatogram (4-OHTOR-diSG 1−4). They all gave identical protonated molecules at *m*/*z* 998 (15% rel intensity) $[M + H]^+$. A doubly charged ion at *m*/*z* 500 $[M + 2H]^{2+}$ was also observed in higher abundance (100%) that had the same retention time as the ion at *m*/*z* 998. On the basis of the qualitative comparison between the total peak area of GSH conjugates formed from the

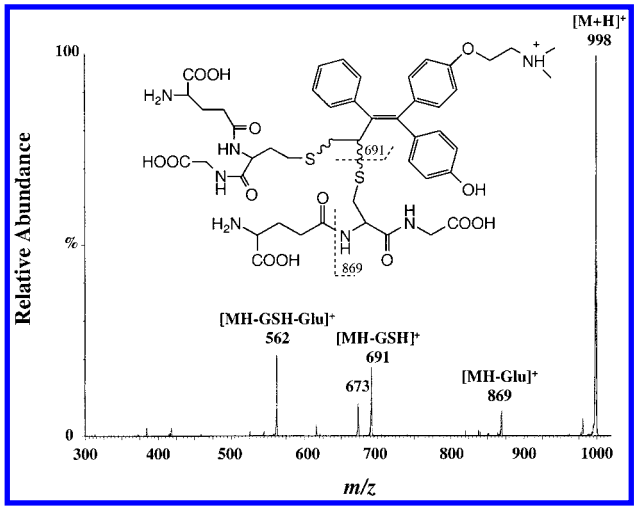


Figure 5. MS/MS fragmentation pattern of the 4-hydroxytoremifene−di-GSH conjugate.

quinone methide and the total peak area of unreacted 4-hydroxytoremifene, the chemical oxidation was 30% efficient. Tandem MS showed loss of 129 mass units (pyroglutamic acid) from the protonated molecule at *m*/*z* 998 (Figure 5). In addition, the product ion spectra of the ion at *m*/*z* 998 also showed the loss of one GSH molecule (*m*/*z* 691) and another loss of a pyroglutamic acid moiety from the second GSH molecule (*m*/*z* 562).

Oxidation of 4-Hydroxytamoxifen and 4-Hydroxytoremifene by Cytochrome P450. Oxidation of 4-hydroxytamoxifen to its quinone methide by cytochrome P450 has been proposed as one of the tamoxifen bioactivation pathways (*20*−*22*). P450 3A4 has been shown to oxidize tamoxifen and its analogues to their corresponding phase I metabolites in humans (*50*). In this study, we used microsomes isolated from rats treated with dexamethasone to induce P450 3A isozymes. In the absence of GSH, we were able to detect the presence of the 4-hydroxytamoxifen quinone methide generated from microsomal incubations. It had the same retention time and mass spectra as the standard 4-hydroxytamoxifen quinone methide. This was surprising since several studies in the past have indicated that most quinone methides dimerize or disproportionate, undergo hydrolysis, or alkylate microsomal proteins in the absence of a trapping agent (*37*, *38*, *51*−*53*) which suggests that direct detection of these reactive intermediates would be difficult. These experiments further emphasize the unusual stability of the 4-hydroxytamoxifen quinone methide under physiological conditions. In terms of quantitative work with radiolabeled GSH, after incubation and separation by HPLC, the radiochromatograms gave peaks with retention times and UV spectra identical to those derived from addition of GSH to the synthetic quinone methides (Table 2). Very low levels of GSH conjugates were detected in microsomal incubations with either 4-hydroxytamoxifen or 4-hydroxytoremifene perhaps due to the fact that little quinone methide was formed from these phenols. 4-Hydroxytoremifene was found to be a better substrate for quinone methide formation, generating approximately 4-fold more GSH trapped products than 4-hydroxytamoxifen.

In the 4-hydroxytamoxifen incubations, three other GSH-dependent peaks were detected, which were later identified as GSH conjugates of 3,4-dihydroxytamoxifen-*o*-quinone (Table 2) (*54*). This is not surprising since previous studies have indirectly shown that 4-hydroxytamoxifen can be oxidized to the catechol, 3,4-dihydroxytamoxifen, by microsomes from phenobarbital-induced rats (*18*, *19*). In these studies, 3,4-dihydroxytamoxifen was not isolated directly but rather the formation of a catechol metabolite was assumed from analysis of a monomethylated metabolite using [^{3}H]-*S*-adenosyl-L-methionine and endogenous catechol-*O*-methyltransferase (*18*). As catechols are highly redox active compounds which can be converted by virtually any oxidative enzyme, metal ion, or, in some cases, molecular oxygen to *o*-quinones which react with GSH, it is not surprising that the GSH conjugates of 3,4-dihydroxytamoxifen-*o*-quinone were observed in our experiments. The amount of *o*-quinone-derived GSH conjugates that was produced was considerably lower than that reported using the methylation to trap the catechol (*18*), perhaps due to differences in the inducing agent as well as other variations in experimental protocols. No additional GSH-dependent peaks were observed in corresponding incubations with 4-hydroxytoremifene which suggests that two-electron oxidation to the quinone methide instead of aromatic hydroxylation and oxidation to an *o*-quinone is the preferred bioactivation pathway. However, synthesis of 3,4-dihydroxytoremifene, the corresponding *o*-quinone, and GSH conjugates will be necessary before the *o*-quinone pathway can be discounted for 4-hydroxytoremifene.

Incubation of 4-Hydroxytamoxifen and 4-Hydroxytoremifene in MCF-7 Cells. Treating a human breast cancer cell line (MCF-7) containing the estrogen receptor with antiestrogens provides a better and more accurate in vitro model system compared to microsomal incubations. Hence, it was selected as our model system for investigating whether quinone methide GSH conjugates are formed. However, several attempts to detect by UV the presence of GSH conjugates in MCF-7 cells treated with either 4-hydroxytamoxifen or 4-hydroxytoremifene failed. We have validated our method by treating the cells with 3,4-dihydroxytamoxifen, another metabolite of tamoxifen, and its GSH conjugates were detected (*54*). This suggests that very little quinone methide is formed in MCF-7 cells treated with either 4-hydroxytamoxifen or 4-hydroxytoremifene and that the UV detection method was not as sensitive as the radiolabeled GSH used in microsomal incubations.

In conclusion, we have shown that the 4-hydroxylated metabolites of tamoxifen and toremifene are both oxidized to unusually stable quinone methides. These electrophiles do react with GSH to form conjugates; however, the rate of reaction is relatively slow compared to those of most *p*-quinone methides (*25*), and the conjugates that are formed are not stable. In fact, the addition of GSH to the tamoxifen quinone methide appears to be a reversible reaction which suggests that little GSH depletion resulting in toxicity would occur in vivo. The toremifene quinone methide forms di-GSH conjugates likely through a mechanism involving formation of an episulfonium ion intermediate, and it is possible that this additional electrophile contributes to the potential toxicity of toremifene in vivo. Finally, we have shown that very little quinone methide GSH conjugate was detected from either phenol in microsomal incubations, and none were observed in MCF-7 cells. These data suggest that the slow rate of formation of the quinone methides from tamoxifen and toremifene, their relatively long lifetimes,

and the instability of the tamoxifen−GSH conjugates may indicate that the quinone methide pathway may play a lesser role in the carcinogenic mechanism of tamoxifen and toremifene. These conclusions are supported by recent reports which have suggested that quinone methide formation from tamoxifen may not be a major pathway in the formation of tamoxifen-derived DNA adducts in rat models (*20*, *21*). Instead, α-hydroxylation of tamoxifen, sulfate ester formation, followed by generation of the tamoxifen carbocation may represent the principle carcinogenic pathway (*15*−*17*). Alternatively, we have shown that 4-hydroxytamoxifen can be converted to the 3,4-dihydroxytamoxifen-*o*-quinone which could represent another potential cytotoxic pathway which has received little attention (*18*, *19*). This possibility is explored in ref *54*.

Acknowledgment. We acknowledge Dr. Richard B. van Breemen, Dr. Dejan Nikolic, and Mr. Lixin Shen for assistance with mass spectrometry experiments and Hewlett-Packard for providing the LC/MS instrumentation. We also thank Dr. Emily Pisha for assistance with the cell culture experiments. This work was supported by NIH Grant CA79870.

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TX990144V