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UTERINE PEROXIDASE CATALYZED FORMATION OF DI-QUINONE METHIDES FROM THE SELECTIVE ESTROGEN RECEPTOR MODULATORS (SERMS) RALOXIFENE AND DESMETHYLATED ARZOXIFENE

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Abstract

Long term usage of the selective estrogen receptor modulator (SERM) tamoxifen has been associated with an increased risk of endometrial cancer. One potential mechanism of tamoxifen induced carcinogenesis involves metabolism to reactive intermediates, such as an *o*-quinone, quinone

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¹Abbreviations:

COATag	covert oxidatively activated tag
DMA	desmethylated arzoxifene
DTT	dithiothreitol
ECL	enhanced chemiluminescence
GPNA	glutamyl <i>p</i> -nitroaniline
γ-GT	γ-glutamyl transpeptidase
HRP	horseradish peroxidase
LC-MS-MS	liquid chromatography-tandem mass spectrometry
MOPS	3-(N-morpholino)propanesulfonic acid
PVDF	polyvinylidene difluoride membrane
Ral	raloxifene
TBST	tris-buffered saline with 0.05% Tween-20.

methide, and carbocation. We have previously shown that the benzothiophene SERMs, raloxifene and desmethylated arzoxifene (DMA), can also be bioactivated to electrophilic quinoids by rat/human liver microsomes and rat hepatocytes. Since the uterus is a major target tissue of estrogens and antiestrogens, it was of interest to determine if quinoids could be formed from SERMs in uterine tissue potentially producing cytotoxic effects. Incubations with rat uterine microsomes showed that both raloxifene and DMA could be oxidized to electrophilic di-quinone methides that were trapped as the corresponding GSH conjugates. A new raloxifene GSH-dependent conjugate was identified as raloxifene Cys-Gly that was formed from the hydrolysis of 7-glutathinyl raloxifene by γ -glutamyl transpeptidase. Interestingly, the metabolism of raloxifene and DMA in rat uterine microsomes was not NADPH-dependent and could be inhibited by cyanide and NADPH or enhanced by H_2O_2 . In addition, co-incubations with the peroxidase substrates guaiacol or *o*-phenylenediamine inhibited di-quinone methide GSH conjugate formation from both SERMs. Incubations of raloxifene and DMA with horseradish peroxidase (HRP) were studied as models of the interaction between benzothiophene SERMs and peroxidase. The results showed that HRP could directly oxidize raloxifene and DMA to the corresponding dimers via the formation of phenoxyl radicals in the absence of exogenous hydrogen peroxide. In addition, GSH appears to be involved in multiple peroxidase-catalyzed oxidative metabolic pathways of benzothiophene SERMs. Finally, COATag (Covert Oxidatively Activated Tag) methodology, which involves the utilization of biotin conjugated raloxifene and DMA, was used in order to identify target proteins by affinity chromatography. Incubations of raloxifene and DMA COATags with rat uterine microsomes showed several modified proteins by western blot analysis. The protein modification could be enhanced by the addition of H_2O_2 and decreased by the addition of NADPH suggesting that unlike liver metabolism the formation of quinoids in the uterus could be mediated by uterine peroxidases.

Introduction

Selective estrogen receptor modulators (SERMs) describe a group of therapeutic agents that manifest estrogen receptor agonist activity in some tissues (i.e., bone and the cardiovascular system), but exhibit antagonist activity in others (i.e., breast). Tamoxifen, as the first SERM, has been used for the treatment of hormone-dependent breast cancer since 1971 (1⁻³). In addition to its established chemotherapeutic use, tamoxifen is also of value as a chemopreventive agent in women at high risk for breast cancer (4). However, it has been known for sometime that women treated with tamoxifen have a 3 to 4-fold increased risk for endometrial cancer (5⁻⁷). One potential mechanism of tamoxifen induced carcinogenesis is the generation of reactive intermediates, such as an *o*-quinone, quinone methide, and carbocation, which could cause DNA damage through the formation of DNA adducts (8⁻¹⁵). In addition, the tamoxifen *o*-quinone and quinone methide have been implicated in alkylation of microsomal proteins (16) which suggests that modification of crucial protein targets could contribute to tamoxifen toxicity. Alternatively, protein modification is known to be complex and could lead to induction of detoxification enzymes such as GSTs or NAD(P)H: quinone oxidoreductase (NQO1) (17), which could contribute to the chemopreventive effects of tamoxifen.

The benzothiophene SERM raloxifene (Scheme 1) is in clinical use for the prevention and treatment of postmenopausal osteoporosis (18). Recent results from the breast cancer chemoprevention STAR trial (Study of Tamoxifen and Raloxifene) indicated that the regular use of raloxifene worked just as well as tamoxifen in reducing breast cancer risk, but with a better safety profile (19), suggesting raloxifene might be a better SERM for cancer chemoprevention. Another benzothiophene SERM, arzoxifene has been reported to have more favorable therapeutic profile for the treatment of hormone dependent cancers as compared to raloxifene (20⁻²²). However, it has previously been shown that both raloxifene and the active metabolite of arzoxifene, desmethylated arzoxifene (DMA, Scheme 1), can be metabolized to

electrophilic/redox-active quinoids by rat/human liver microsomes and hepatocytes which might cause cytotoxic effects (23-25).

Various SERMs have been demonstrated to be oxidatively bioactivated by P450 enzymes in liver microsomes to quinoid metabolites (15); however, studies in the potential target tissues for SERM carcinogenesis have been less common. Endometrial carcinomas are a common gynecological malignancy of the female urogenital tract (26) and the metabolism of endogenous estrogens to *o*-quinones has been postulated to be a major factor in carcinogenesis (27, 28). Possible enzymes responsible for bioactivation of estrogens and antiestrogens in the endometrium include the P450 enzymes and peroxidases. Within the human endometrium, the known P450 isozymes that are present are CYP 2C9, 3A, 1A1, and 1B1 (29). Peroxidase activity has been detected in uterine tissues of both rodents (30, 31) and humans (32). Levels of peroxidase activity can be substantially elevated by inflammation and by hormonal fluctuations. Treatments with 17 β -estradiol (E₂), diethylstilbestrol (DES) (30, 31), or tamoxifen (10) have been found to increase the level of peroxidase activity in the uterus. Bodell *et al.* have demonstrated that horseradish and uterine peroxidase could activate both 4-hydroxytamoxifen and metabolite E (*cis/trans*-1,2-diphenyl-1-(4-hydroxyphenyl)-but-1-ene) to form DNA adducts (10, 33). These results suggested that uterine peroxidase and P450 found in endometrial tissue could activate SERMs to reactive intermediates that might play a role in endometrial cancer risk.

In the current study, we investigated the potential for raloxifene and DMA to form reactive quinoids in rat uterine microsomes. Surprisingly, the results showed no evidence for bioactivation by P450s. Instead the data showed that uterine peroxidases catalyzed di-quinone methide formation, which was measured by the appearance of GSH- and GSH-related conjugates of raloxifene di-quinone methide which were characterized using LC-MS-MS and NMR. Preliminary evidence was gained for modification of rat uterine microsomal proteins by the di-quinone methide metabolites using covalent oxidized activated tag (COATag) methodology (34).

Materials and Methods

Caution: Raloxifene and DMA di-quinone methides were handled in accordance with the NIH Guidelines for the Laboratory Use of Chemical Carcinogens (35)

All solvents and reagents were purchased from either Aldrich Chemical (Milwaukee, WI), Fisher Scientific (Itasca, IL), or Sigma (St. Louis, MO) unless stated otherwise. Solutions of guaiacol and H₂O₂ were prepared fresh daily. H₂O₂ concentration was determined spectrophotometrically (240 nm). ECL was obtained from Amersham Biosciences (Piscataway, NJ). NuPAGE precast gels and XCell SureLock™ Mini-Cell were obtained from Invitrogen (Carlsbad, CA). DMA, 4'-DMA, and compound **1** (Scheme 2) were prepared by a previously described literature procedure (24) as was the raloxifene COATag (34).

Instrumentation

¹H, ¹³C -NMR spectra were obtained with Bruker Ultrashield 400, Avance 300, or DRX500 spectrometers. UV spectra were obtained on a Hewlett-Packard (Palo Alto, CA) 8452A photodiode array UV-Vis spectrophotometer. LC-MS-MS was carried out using an 1100 Agilent (Palo Alto, CA) LC/MSD ion trap mass spectrometer equipped with an electrospray ionization source under positive ion detection mode.

HPLC

Four methods were used to analyze and separate metabolites and conjugates. The composition of HPLC solvent for each method is the mixture of two solutions, solution A: water with 10%

methanol and 0.1% formic acid; solution B: acetonitrile with 0.1% formic acid. *Method A*: Analytical HPLC analysis of raloxifene di-quinone methide GSH conjugates was performed using an Agilent Zorbax Rx-C₈ column (4.6 × 250 mm, 5 μm) with UV absorbance detection at 284 nm and MS-MS analysis. The mobile phase consisted of a linear gradient of solution B from 10 - 40% over 20 min, 5 min gradient of solution B from 40 - 90% at a flow rate of 1 mL/min. *Method B*: Analytical HPLC analysis of DMA di-quinone methide GSH conjugates was performed using an Ultrasphere C₁₈ column (4.6 × 250 mm, 5 μm) with UV absorbance detection at 316 nm and was used for LC-MS-MS analysis. The mobile phase consisted of a linear gradient of solution B from 10 - 30% over 20 min, 15 min gradient of solution B from 30 - 90% at a flow rate of 1 mL/min. *Method C*: Semi-preparative HPLC was used to isolate conjugates for additional characterization. Separation was carried out using an Ultrasphere C₁₈ column (10 × 250 mm) and a mobile phase consisting of a 40 min linear gradient of solution B from 10 - 40% at a flow rate of 3 mL/min. *Method D*: the analytical HPLC analysis of the incubations of raloxifene/DMA with HRP was performed using an Agilent Eclipse XDB-C₁₈ column (4.6 × 250 mm, 5 μm) with UV absorbance detection at 316 nm and MS-MS analysis. The mobile phase consisted of a linear gradient of solution B from 10 - 30% over 20 min, 20 min gradient of solution B from 30 - 60%, and then 60 - 90% of solution B over 5 min at a flow rate of 1 mL/min.

Synthesis of DMA COATag

The DMA COATag synthesis is shown in Scheme 2. 6-Methoxy-3-(4-benzyloxyphenoxy)-2-(4-methoxyphenyl) benzo[b]thiophene *S*-oxide (**2**). 4-Benzyloxyphenol (330 mg, 1.65 mmol) was dissolved in 5 mL anhydrous DMF, NaH 60 mg (60%, dispersed in oil) was slowly added followed by the addition of compound **1** (550 mg, 1.5 mmol) and the reaction mixture was stirred at room temperature for 1 h. The mixture was diluted with 100 mL ethyl acetate, the solution was washed with water, and the aqueous phase was extracted with 20 mL CH₂Cl₂, combined with ethyl acetate, and dried over anhydrous MgSO₄. The solvent was removed *en vacuo* giving compound **2** as a yellow solid (500 mg, 95%). ¹H-NMR (DMSO-d₆, 300 MHz): δ 7.65-7.72 (m, 3H), 7.34-7.43 (m, 5H), 6.96-7.09 (m, 8H), 5.03 (s, 2H), 3.86 (s, 3H), 3.77 (s, 3H). ¹³C-NMR (DMSO-d₆, 75 MHz): δ 160.4, 159.3, 154.6, 148.8, 147.6, 144.2, 136.9, 131.9, 129.1, 128.4, 127.8, 127.7, 125.8, 123.2, 121.7, 117.9, 117.5, 116.0, 114.5, 112.7, 69.6, 56.1, 55.2. 6-Methoxy-3-(4-benzyloxyphenoxy)-2-(4-methoxyphenyl) benzo[b]thiophene (**3**). Compound **2** (1.0 g, 2 mmol) was dissolved in 20 mL anhydrous THF, LiAlH₄ (120 mg, 3.2 mmol) was added, and the reaction mixture was stirred at room temperature for 1 h. The reaction was quenched with concentrated sodium hydroxide solution, the residue was extracted with 2 × 100 mL ethyl acetate, and dried over anhydrous MgSO₄. The solvent was removed and the residue was purified by column chromatography (4:1, hexanes/ethyl acetate) to give compound **3** as a slightly yellow solid (665 mg, 91%). ¹H-NMR (DMSO-d₆, 400 MHz): δ 7.64 (d, 2H, *J* = 8.8 Hz), 7.56 (d, 1H, *J* = 2.2 Hz), 7.31-7.42 (m, 5H), 7.19 (d, 1H, *J* = 8.8 Hz), 6.86-7.00 (m, 7H), 4.99 (s, 2H), 3.82 (s, 3H), 3.75 (s, 3H). ¹³C-NMR (DMSO-d₆, 100 MHz): δ 158.9, 157.7, 153.7, 151.1, 139.2, 137.0, 136.1, 128.4, 128.2, 127.8, 127.7, 127.3, 125.8, 124.1, 121.5, 116.0, 115.9, 114.7, 114.5, 106.0, 69.6, 55.5, 55.1. 6-Methoxy-3-(4-hydroxyphenoxy)-2-(4-methoxyphenyl)benzo[b]thiophene (**4**). Compound **3** (4.7 g, 10 mmol) was dissolved in 100 mL acetonitrile, sodium iodide (2.2 g, 15 mmol) was added to this solution followed by 6.3 mL BF₃ etherate (50 mmol), and the reaction mixture was stirred at room temperature for 8 h. Sodium iodide (1 g) and 1.5 mL BF₃ etherate were added and stirred for 2 h. The solution was diluted with CH₂Cl₂ (500 mL), washed with aqueous sodium bis-sulfite solution and brine, and dried over anhydrous MgSO₄. The solvent was removed and the residue was purified by column chromatography (3:1, hexanes/ethyl acetate), to give compound **4** as a white solid (3.58 g, 94%). ¹H-NMR (acetone-d₆, 300 MHz): δ 8.05 (s, 1H), 7.69 (d, 2H, *J* = 8.7 Hz), 7.43 (d, 1H, *J* = 1.7 Hz), 7.26 (d, 1H, *J* = 8.8 Hz), 6.74 - 6.96 (m, 7H), 3.86 (s, 3H), 3.79 (s, 3H). ¹³C-NMR (acetone-d₆, 75 MHz): δ 160.4, 159.2, 153.6, 151.8, 141.0, 137.8, 129.5, 129.1, 127.1,

125.9, 122.9, 117.2, 117.0, 115.4, 115.2, 106.5, 56.1, 55.7. *6-Methoxy-3-[4-(2-bromoethoxy)phenoxy]-2-(4-methoxyphenyl) benzo[b]thiophene (5)*. Compound **4** (3.5 g, 9.2 mmol) was mixed with 1,2-dibromoethane (50 mL), 10 mL anhydrous THF, and KOH (1.2 g, 18.4 mmol, 87%). The reaction mixture was heated at 80 °C for 8 h. The solution was diluted with 200 mL chloroform, washed with water and brine, the solvent was removed, and the residue purified by column chromatography (7:1, hexanes/ethyl acetate), to give compound **5** as a white solid (4.0 g, 89%). ¹H-NMR (acetone-d₆, 300 MHz): δ 7.68 - 7.70 (m, 2H), 7.45 (d, 1H, *J* = 2.1 Hz), 7.26 (d, 1H, *J* = 8.8 Hz), 6.90-6.97 (m, 7H), 4.28 (t, 2H, *J* = 5.5 Hz), 3.87 (s, 3H), 3.80 (s, 3H), 3.72 (t, 3H, *J* = 5.8 Hz). ¹³C-NMR (acetone-d₆, 75 MHz): 160.5, 159.3, 154.7, 153.0, 140.6, 137.8, 129.5, 128.9, 127.3, 125.7, 122.8, 117.2, 116.8, 115.5, 115.2, 106.5, 69.5, 56.0, 55.7, 31.1. *6-Methoxy-3-(4-[2-(1-(4-(2-azidoethyl)piperidinyl)ethoxy]phenoxy)-2-(4-methoxyphenyl) benzo[b]thiophene (6)*. Compound **5** (2.4 g, 4.9 mmol), 4-(2-azidoethyl)piperidine (2.6 g, 9.8 mmol), K₂CO₃ (2.7 g, 19.8 mmol), and anhydrous acetonitrile (40 mL) were mixed together and the reaction mixture was refluxed overnight. After filtration through Celite and concentration, the residue was purified by column chromatography (7:1, CH₂Cl₂/MeOH) to give compound **6** as a white solid (2.1 g, 72%). ¹H-NMR (acetone-d₆, 400 MHz): δ 7.68 (d, 2H, *J* = 8.9 Hz), 7.41 (d, 1H, *J* = 2.1 Hz), 7.24 (d, 1H, *J* = 8.8 Hz), 6.80-6.92 (m, 7H), 3.96 (t, 2H, *J* = 5.9 Hz), 3.84 (s, 3H), 3.76 (s, 3H), 3.32 (t, 2H, *J* = 7.1 Hz), 2.88-2.91 (m, 2H), 2.63 (t, 2H, *J* = 5.7 Hz), 1.95-2.03 (m, 2H), 1.59-1.63 (m, 2H), 1.45-1.50 (m, 2H), 1.29-1.33 (m, 1H), 1.15-1.22 (m, 2H). ¹³C-NMR (acetone-d₆, 100 MHz): 160.3, 159.0, 155.2, 152.4, 140.6, 137.6, 129.3, 128.0, 127.1, 125.6, 122.7, 117.0, 116.3, 115.3, 115.1, 106.4, 67.4, 58.2, 55.9, 55.5, 54.9, 49.5, 35.9, 33.9, 32.9. *6-Hydroxy-3-(4-[2-(1-(4-(2-azidoethyl)piperidinyl)ethoxy]phenoxy)-2-(4-hydroxyphenyl) benzo[b]thiophene (7)*. Compound **6** (300 mg, 0.54 mmol) was dissolved in 5 mL CH₂Cl₂, treated with 1N HCl/ether (2 mL) for 15 min, and the solvent was removed en vacuo. The residue was dissolved in 15 mL CH₂Cl₂ and BF₃·SMe₂ (2 mL) was added. The reaction mixture was stirred at room temperature for 6 h and poured into saturated aqueous NaHCO₃ solution. The mixture was extracted with ethyl acetate, concentrated, and purified by column chromatography (5:1, CH₂Cl₂/MeOH), to give compound **7** as a white solid (235 mg, 82%). ¹H-NMR (DMSO-d₆, 400 MHz): δ 9.74 (bs, 2H), 7.49 (d, 2H, *J* = 8.7 Hz), 7.25 (d, 1H, *J* = 1.9 Hz), 7.08 (d, 1H, *J* = 8.6 Hz), 6.78-6.86 (m, 7H), 3.95 (t, 2H, *J* = 5.7 Hz), 3.33 (t, 2H, *J* = 7.0 Hz), 2.85-2.88 (m, 2H), 2.61 (t, 2H, *J* = 5.7 Hz), 1.95 (m, 2H), 1.57-1.60 (m, 2H), 1.41-1.46 (m, 2H), 1.25-1.28 (m, 1H), 1.08-1.17 (m, 2H). ¹³C-NMR (DMSO-d₆, 100 MHz): δ 157.3, 155.7, 153.7, 151.0, 138.9, 135.9, 128.2, 126.3, 125.1, 122.7, 121.5, 116.0, 115.8, 115.5, 114.8, 108.0, 65.9, 57.0, 53.6, 48.3, 34.7, 32.6, 31.5. *6-Hydroxy-3-(4-[2-(1-(4-(2-(1-biotinyl-aminocaproylethyl)piperidinyl)ethoxy]phenoxy)-2-(4-hydroxyphenyl) benzo[b]thiophene (8)*. Compound **7** (40 mg, 0.075 mmol), THF (2 mL), PPh₃ (30 mg, 0.12 mmol), and H₂O (20 μL) were mixed together and stirred overnight. After concentration, biotin-LC succinimide active ester (34 mg, 0.075 mmol) was added followed by DIPEA (20 μL), and the reaction mixture was stirred overnight. Compound **8** was directly purified by column chromatography (3:1 CH₂Cl₂/MeOH and 5% ammonium hydroxide), to give a white solid (46 mg, 73%). ¹H-NMR (DMSO-d₆, 400 MHz): δ 9.65 (bs, 2H), 7.66-7.74 (m, 2H), 7.49 (d, 2H, *J* = 8.7 Hz), 7.24 (d, 1H, *J* = 1.8 Hz), 7.06 (d, 1H, *J* = 8.7 Hz), 6.77-6.86 (m, 7H), 6.43 (s, 1H), 6.36 (s, 1H), 4.27-4.31 (m, 1H), 4.10-4.12 (m, 1H), 3.95 (t, 2H, *J* = 5.8 Hz), 2.97-3.10 (m, 5H), 2.78-2.87 (m, 3H), 2.55-2.61 (m, 3H), 1.95-2.05 (m, 4H), 1.93 (t, 2H, *J* = 10.7 Hz), 1.07-1.60 (m, 20H). ¹³C-NMR (DMSO-d₆, 100 MHz): δ 171.8, 171.7, 162.7, 157.3, 155.7, 153.7, 151.0, 138.9, 135.9, 128.2, 126.3, 125.1, 122.7, 121.5, 116.0, 115.8, 115.5, 114.8, 108.0, 65.9, 61.0, 59.2, 57.1, 55.4, 53.8, 39.8, 38.3, 35.9, 35.3, 35.2, 32.7, 31.8, 30.7, 29.0, 28.2, 28.0, 26.1, 25.3, 25.1.

Preparation of Rat Uterine Microsomes

Female Sprague Dawley rats (25 rats, 200 - 220 g) were obtained from Sasco Inc. (Omaha, NE). The rats were sacrificed and the entire uterus from oviduct to cervix was removed,

dissected free of fat and connective tissue, and washed with cold sucrose buffer then weighed. The uteri were minced and transferred into cold homogenizer tubes with 2/3 full 0.25 M sucrose, 0.1 mM EDTA, pH 7.4, buffer (3 mL/g tissue). The uterine pieces were homogenized using a polytron homogenizer until no more uterine particles could be observed. The homogenate was then transferred to cold sorval tubes, balanced, and centrifuged for 20 min at 12,000 g at 4 °C. The supernatant was transferred to ultracentrifuge tubes, balanced, and centrifuged at 110,000 g for 90 min at 4°C. The supernatant was aspirated from each tube and the remaining microsomal pellets were re-suspended in 50 mM K₂HPO₄, 170 mM KCl buffer containing protease inhibitors (20 ng/mL pepstatin and 20 ng/mL leupeptin) in cold homogenizer tubes and homogenized until pellets were liquefied. The suspension was centrifuged for 60 min at 110,000 g at 4°C, the supernatant was aspirated, and the microsomal pellets were re-suspended as described above except that the buffer did not contain inhibitors. Protein concentration was determined using the BioRad protein assay kit (Hercules, California) and P450 concentrations was determined as described previously (36). The measurement of peroxidase activity were performed by modification of the method of Saito *et al.* (37). The rate of oxidation of guaiacol was measured at room temperature. The standard assay system contained 33 mM guaiacol, 0.27 mM hydrogen peroxide, and 50 mM phosphate buffer, pH 7.4, in a total volume of 2 mL. The reaction was initiated by the addition of hydrogen peroxide (20 µL), and initial rates of guaiacol oxidation were determined from the increase in absorbance at 470 nm for the first minute. The amount of enzyme was adjusted to ensure that the initial rate was zero order. An enzyme unit ($\epsilon = 5.57 \times 10^3 \text{ cm}^{-1}\text{M}^{-1}$) was defined as the amount of enzyme required to produce an increase of 1 absorbance unit/min under the assay conditions. The rat uterine peroxidase activity was 5.2 ± 0.6 units/mg protein.

Metabolism of Raloxifene and DMA by Rat Uterine Microsomes in the Presence of GSH

A solution containing the substrate (30 µM), rat uterine microsomes (1 mg/mL), GSH (0.6 mM), 0.5 mM H₂O₂ or a NADPH-generating system (including 1.0 mM NADP⁺, 5.0 mM MgCl₂, 5.0 mM isocitric acid, and 0.2 unit/mL isocitric dehydrogenase), in 50 mM phosphate buffer (pH 7.4, 1 mL total volume) was incubated for 30 min at 37 °C. For control incubations, NADP⁺ was omitted. The reactions were terminated by chilling in an ice bath followed by the addition of perchloric acid (50 µL/mL). The reaction mixtures were centrifuged at 16,000 g for 5 min, and the solutions were extracted using PrepSep™ C₁₈ solid-phase extraction cartridges, eluted with methanol, and concentrated to a final volume of 250 µL. Aliquots (50 µL) were analyzed directly using LC-MS-MS with HPLC method A for raloxifene and method B for DMA.

Synthesis and characterization of Ral-CG

A solution containing raloxifene (50 µM), HRP type IV (8 unit/mL), GSH (0.5 mM), with or without γ -glutamyl transpeptidase (γ -GT, 2 unit/mL), with or without Cys-Gly (500 µM) in 50 mM phosphate buffer (pH 7.4, 1 mL) was incubated for 30 min at 37 °C, and subsequently processed as described above. HPLC method C was used for the separation of Ral-CG on a semi-preparative HPLC column. Ral-CG: ¹H-NMR (500 MHz, DMSO-*d*₆): δ 8.2 (s, 1H), 7.66 (d, *J* = 8.5 Hz, 2H), 7.25 (d, *J* = 8.2 Hz, 1H), 7.18 (m, 3H), 6.91 (d, *J* = 8.5, 2H), 6.67 (d, *J* = 8.3 Hz, 2H), 4.08 (m, 2H), 3.59 (s, 2H), 3.17 (m, 1H), 2.85 (m, 2H), 2.68 (m, 4H), 2.61 (m, 2H), 1.45 (m, 5H), 1.35 (m, 1H); positive ion electrospray MS *m/z* 326 (100%) [*M* + 2H]²⁺.

γ -Glutamyl Transpeptidase Assay

Assays were performed by modification of the method of Storozhenko *et al.* (38). The release of *p*-nitroaniline was monitored at room temperature at 405 nm. The standard assay system contained 50 mM phosphate buffer (pH, 7.4), 1 mM glutamyl *p*-nitroaniline (GPNA), 40 mM Gly-Gly, and 0.06 mg/mL uterine microsomes, in a total volume of 2 mL. The initial rates of

p-nitroaniline release were determined from the increase in absorbance at 405 nm for the first minute. The amount of enzyme was adjusted to ensure that the initial rate was as close as possible to zero order. An enzyme unit ($\epsilon = 8,800 \text{ cm}^{-1}\text{M}^{-1}$) was defined as the amount of enzyme required to produce an increase of 1 absorbance unit/min under the assay conditions. The activity of γ -GT in rat uterine microsomes was determined to be 0.15 ± 0.05 units/mg protein.

Inhibition of SERM Metabolism in Uterine Microsomal Incubations

The incubation mixture consisted of rat uterine microsomes (1 mg/mL), 30 μM raloxifene or DMA, 33 mM guaiacol or 20 mM *o*-phenylenediamine, 500 μM GSH, and 0.27 mM (in guaiacol experiment) or 2 mM (in *o*-phenylenediamine experiment) H_2O_2 in a total volume of 1 mL phosphate buffer (50 mM, pH 7.4). The reactions were carried out for 30 min at 37 °C. Control studies were performed without the addition of guaiacol or *o*-phenylenediamine, subsequently processed as described above. Similarly, NADPH (1 mM), CN^- (100 μM), or H_2O_2 (10 μM or 100 μM) were included to evaluate their effect on the formation of the corresponding GSH conjugates.

Oxidation of Raloxifene and DMA by Horseradish Peroxidase (HRP)

A solution containing the substrate (5 μM), HRP type IV (8 unit/mL), with or without GSH (500 μM), with or without catalase (1000 unit/mL or 4000 unit/mL), with or without H_2O_2 (10 μM or 100 μM) in 50 mM phosphate buffer (pH 7.4, 0.5 mL total volume) was incubated for 30 min at 37 °C. The reactions were terminated by chilling in an ice bath followed by the addition of 0.5 mL mixture of methanol and acetonitrile (1:1) containing perchloric acid (50 $\mu\text{L/mL}$) and 4'-F-DMA (5 μM , as internal standard). The reaction mixtures were centrifuged at 12,000 g for 15 min and aliquots of the supernatant (100 μL) were analyzed by LC-MS using method D described above.

Modifications of Rat Uterine Microsomal Proteins by Raloxifene COATag and DMA COATag

A solution containing the raloxifene COATag or DMA COATag (30 μM), rat uterine microsomes (1.5 mg/mL), was incubated with or without a NADPH generating system (5.0 mM isocitric acid, 0.2 unit/mL isocitric acid dehydrogenase, and 1.0 mM NADP^+), with or without H_2O_2 (1 mM), in 50 mM phosphate buffer (pH 7.4, 100 μL total volume). The reaction was quenched with DTT (1 mM) and the mixture was loaded directly into NuPAGE for electrophoresis. The modified proteins were separated by 4-12% NuPAGE gel with MOPS running buffer. The gels were transferred onto PVDF membranes using XCell SureLock™ Mini-cell according to the manufacture's instructions. The membranes were blocked with 5% milk in $0.5 \times \text{TBST}$ for 1 h at room temperature, overnight at 4 °C, followed by washing the membranes three times at 5 min intervals. The membranes were incubated with HRP-conjugated streptavidin (1:20,000 dilution) for 1 h followed by washing the membranes three times at 5 min intervals. The membrane was incubated with ECL for 5 min at room temperature. The membranes were exposed to film for 10 s to 5 min before development.

Results

Oxidation of Raloxifene and DMA in Rat Uterine Microsomes

In incubations of raloxifene with rat uterine microsomes in the presence of GSH (Figure 1A), three mono-GSH conjugates were obtained (Figure 2), that were identical to those previously observed in experiments with rat or human liver microsomes (23, 25). Similar uterine incubations with DMA in the presence of GSH (Figure 1B), also gave the same GSH conjugates to those previously observed in liver microsomes (24). It is important to emphasize that, in contrast to the studies in liver microsomes, in uterine microsomes the raloxifene and DMA

GSH conjugates were formed in the absence of NADPH, suggesting a P450-independent bioactivation mechanism.

Interestingly, in the uterine microsomes, in addition to the three known raloxifene GSH conjugates, one new GSH-dependent raloxifene conjugate was observed which was identified as Ral-Cys-Gly (Ral-CG) by LC-MS-MS and $^1\text{H-NMR}$. It is likely that Ral-CG resulted from hydrolysis of the major raloxifene GSH conjugate (Ral-SG3), catalyzed by γ -glutamyl transpeptidase (γ -GT), which is known to have activity in uterine tissues (39, 40). Using glutamyl *p*-nitroaniline (GPNA) as a substrate, γ -GT activity was determined to be 0.15 ± 0.05 units/mg protein in rat uterine microsomes (38).

In order to confirm the mechanism of Ral-CG formation, model systems were set up using HRP and γ -GT. Incubations with purified HRP, raloxifene, and GSH gave the three raloxifene GSH conjugates observed in the uterine incubations (Figure 2A). However, if Cys-Gly was used as the trapping agent instead of GSH the Ral-CG conjugate was detected (Figure 2B). Persuasively, when raloxifene was incubated with HRP, GSH, and γ -GT, the Ral-CG conjugate was again detected (Figure 2C).

Inhibition of Peroxidase-catalyzed Oxidation of Raloxifene and DMA in Rat Uterine Microsomes

The lack of dependence on NADPH on bioactivation of raloxifene and DMA in uterine microsomes implied that the oxidation was not mediated by P450. Indeed, the addition of NADPH (1 mM) to the incubation mixture inhibited the formation of raloxifene and DMA GSH conjugates by 50% and 80%, respectively (Figure 3), likely due to the known ability of NAD(P)H directly to reduce quinoids. Therefore a role for alternative oxidative enzymes was examined. The well-known peroxidase activity in uterine tissue inspired the study of the effects of several peroxidase inhibitors. The addition of CN^- (100 μM) resulted in 90% and 80% inhibition of di-quinone methide GSH conjugate formation from raloxifene and DMA, respectively, indicating the involvement of heme iron in the oxidation process implying a role for peroxidase (Figure 3). Addition of guaiacol and *o*-phenylenediamine, peroxidase inhibitors which are readily oxidized by peroxidases in the presence of H_2O_2 (37), completely inhibited the formation of GSH conjugates in rat uterine microsomes (data not shown). These data strongly implicate uterine peroxidase in catalysis of the observed oxidative bioactivation of the benzothiophene SERMs. Oxidation of raloxifene and DMA was slightly increased by the addition of 0.5 mM H_2O_2 (Figure 3); however, the very modest effect of H_2O_2 stimulated further mechanistic experiments.

Oxidation of Raloxifene and DMA by Horseradish Peroxidase (HRP)

HRP was used as a model peroxidase enzyme to further explore the oxidation mechanism of the benzothiophene SERMs. No evidence was observed for oxidation of the SERMs by H_2O_2 (10 - 500 μM) in the absence of HRP (data not shown). In contrast, HRP incubations of raloxifene (Figure 4A) or DMA (data not shown) in the absence of H_2O_2 gave the corresponding dimers which were characterized by mass spectrometry [raloxifene dimer, m/z 473 ($\text{M} + \text{H}^+$) $^{2+}$; DMA dimer, m/z 461 ($\text{M} + \text{H}^+$) $^{2+}$]. The exact structures of the dimers are not known; however, based on similar experiments with 4-hydroxytamoxifen (41) the structures shown in Scheme 1 are reasonable predictions. Addition of H_2O_2 to the HRP incubations dramatically increased the formation of dimer (Figure 4B). Formation of the raloxifene dimer has been reported previously in the presence of an oxidizing solution of NO, on reaction with peroxynitrite, and in solutions of $\text{NO}^-/\text{H}_2\text{O}_2$ and either HRP or FeCl_3 (42).

In the absence of H_2O_2 , incubations with HRP, raloxifene, and GSH led to the formation of raloxifene di-quinone methide GSH conjugates as major metabolites (Figure 5A), and under

these conditions, dimers were not observed. Raloxifene (and DMA) turnover was also significantly increased as compared with HRP incubations in the absence of GSH and H₂O₂ (Figure 6). Furthermore, the addition of H₂O₂ (10 μ M, 100 μ M) to the incubation of raloxifene with HRP and GSH also increased the consumption of raloxifene by HRP (Figure 5C), and it was interesting to note, that metabolite formation was highly dependent on the concentration of H₂O₂ (Figure 5B, 5C). At relatively low H₂O₂ concentrations (10 μ M), both Ral-GSH conjugates and raloxifene dimer were observed (Figure 5B) whereas at higher concentrations (100 μ M, Figure 5C) the raloxifene dimer was the major product likely due to oxidation of GSH to GSSG.

Catalase converts H₂O₂ to oxygen and H₂O, and as a result, the addition of catalase usually inhibits peroxidase oxidation by removing H₂O₂. However, the above observations showed that H₂O₂ was not required for HRP mediated oxidation of raloxifene, therefore this expectation is not so clear cut. In fact, in this system, the addition of catalase to the incubation mixture containing raloxifene, HRP, and GSH increased the formation of GSH conjugates and increased the consumption of raloxifene (Figure 7B, 7C). An explanation for these observations is that catalase addition led to removal of the H₂O₂ formed in the reaction medium containing HRP, GSH, and raloxifene, which otherwise would oxidize GSH.

Alkylation of Rat Uterine Microsomal Proteins by Raloxifene and DMA COATags

Since raloxifene and DMA were converted to electrophilic di-quinone methides catalyzed by rat uterine microsomes, it was of interest to determine if these electrophiles could covalently modify uterine microsomal proteins. Biotinylated raloxifene and DMA were incubated with rat uterine microsomes at physiological pH and temperature for 30 min, and then alkylated microsomal proteins were isolated by affinity chromatography, separated, and visualized with streptavidin-peroxidase based detection systems. The results showed that both raloxifene and DMA COATags were oxidized to thiol-reactive electrophiles which modified liver and uterine microsomal proteins (Figure 8). Because of the existence of the phenolate leaving group which could make some of the modified proteins by DMA COATag undetectable (24), less protein modification was observed with the DMA COATag as compared to raloxifene COATag in both rat liver and uterine microsomal incubations. Similar to what was observed with the GSH conjugates, the addition of NADPH (1 mM) decreased protein modification whereas the addition of H₂O₂ (0.5 mM) enhanced the protein alkylation. These data provide further evidence that SERM electrophilic di-quinone methides formed by uterine peroxidases have the ability to alkylate microsomal proteins.

Discussion

It has been reported that tamoxifen could be bioactivated by uterine microsomes to reactive metabolites that might play a role in endometrial cancer risk (10). Incubations of rat uterine microsomes with raloxifene and DMA showed that both SERMs were oxidized to electrophilic di-quinone methides, which were trapped as GSH conjugates. Similar GSH conjugates were previously reported in liver microsomal incubations (22, 23); however, in the uterine microsomes, these oxidations were not P450 catalyzed and instead appeared to be mediated by uterine peroxidases. In support of this observation, di-quinone methide GSH conjugate formation was inhibited by the addition of NADPH, cyanide, and peroxidase competitive inhibitors (guaiacol and *o*-phenylenediamine), and slightly enhanced by the addition of H₂O₂. The formation of a novel di-quinone methide Cys-Gly-adduct was accounted for by γ -glutamyl transferase microsomal activity, which was replicated in a model system.

Generally, peroxidases catalyze the one-electron oxidation of a variety of xenobiotics using hydrogen peroxide or alkylhydroperoxides as oxidant (Scheme 3, mechanism 1) (43). The interaction between H₂O₂ and the protoheme group located at the active site of peroxidases

generates an oxo ferryl protophyrin-cation radical (compound I) which oxidizes the substrate (43). However, oxidation of raloxifene and DMA in uterine microsomes was only modestly enhanced by addition of exogenous H_2O_2 . The lack of a requirement for H_2O_2 in peroxidase mediated oxidations is not unprecedented; in some cases the oxidation of substrates by peroxidases can be H_2O_2 independent. For example, HRP can oxidize indoleacetic acid (44), NADH (45), and epinephrine (46) with no requirement for H_2O_2 . A mechanistic explanation has been proposed by Banerjee *et al.* (46), in which the substrate directly reduces the ferric HRP to the ferrous form, which reacts with oxygen to generate compound III (Scheme 3, mechanism 2). Compound III decomposes to generate the native HRP and superoxide which can spontaneously dismutate to H_2O_2 thus providing a cofactor for mechanism 1. Once H_2O_2 is generated in the system, the normal peroxide mediated oxidation of substrate is initiated due to the higher affinity of ferric HRP toward H_2O_2 .

Although SERM oxidation in uterine microsomes showed no requirement for H_2O_2 , the model peroxidase, HRP, poorly catalyzed oxidation of raloxifene or DMA in absence of GSH. Small amounts of dimer were observed indicative of semiquinone radical formation by HRP under non-catalytic conditions; however, addition of excess H_2O_2 led to catalytic conditions for formation of the semiquinone and the resultant dimer, via the standard peroxidase Mechanism 1 (Scheme 3). Interestingly, addition of excess GSH to the HRP incubation was also observed to increase SERM consumption, but in contrast to the H_2O_2 experiment, GSH conjugates were the only observed products. In the absence of exogenous H_2O_2 , Mechanism 2 provides a pathway for di-quinone methide formation. However, the dependence of this reaction on GSH, indicates a role for GSH in activating HRP as an oxidant, and possibly as a source of H_2O_2 . GSH can itself readily autoxidize (47), or in the presence of HRP compound I and/or II as oxidants, generate superoxide, leading to hydrogen peroxide (48) (Scheme 3). The addition of exogenous H_2O_2 to the HRP/GSH incubations was observed to shift the reaction back to Mechanism 1 by direct oxidation of GSH. Finally, there is evidence for semiquinone formation mediated by HRP and reaction with glutathionyl radicals in model systems (49) leaving the possibility that GSH conjugates may result in part from radical addition and recombination.

The HRP model reactions were able to replicate the observations in uterine microsomes: the formation of a novel Cys-Gly-conjugate and the lack of dependence on exogenous H_2O_2 for the oxidative bioactivation of raloxifene and DMA. The results, in both microsomal and model systems, emphasize the oxidative lability of the benzothiophene SERMs towards formation of di-quinone methides which readily form adducts with nucleophiles such as GSH.

Covalent binding of reactive intermediates to nucleophilic sites in proteins is generally recognized as an initial and crucial event in the etiology of many chemical-induced toxicities (50, 51). Kupfer and colleagues have revealed that tamoxifen can be metabolized by rat and human liver microsomes into reactive intermediates which bind irreversibly to microsomal proteins (16). Our previous studies have shown that raloxifene can be bioactivated by rat liver microsomes into quinoids which covalently modified microsomal proteins using the COATag technology (34). It was of interest to determine if raloxifene and DMA could modify uterine microsomal proteins after bioactivation since the uterus is one of the major target tissues of estrogens and antiestrogens *in vivo* (52). In the current study, by using the biotin conjugated raloxifene and DMA COATags, we observed that both raloxifene and DMA could be oxidized to diquinone methides which further covalently modified rat uterine microsomal proteins (Figure 8). The covalent uterine microsomal protein modification could be enhanced by the addition of H_2O_2 and decreased by the addition of NADPH, which suggests that rat uterine peroxidases catalyze di-quinone methide formation leading to protein alkylation. Future work involving 2D gel electrophoresis and proteomics experiments should allow identification of these uterine target proteins.

In conclusion, raloxifene and DMA can be oxidized by rat uterine microsomes to electrophilic di-quinone methides which can either be trapped by GSH or covalently modify rat uterine microsomal proteins. In contrast to oxidation by liver microsomes, oxidative bioactivation was mediated by uterine peroxidases. These data suggest that di-quinone methides will be formed from benzothiophene SERMs in the uterus. Although at this stage, the exact biological targets and resulting effects of these reactive quinoids are not known, the implication of SERM bioactivation in tissues closely linked to the potential carcinogenicity of SERMs is significant.

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References

- (1). Fisher B, Costantino J, Redmond C, Poisson R, Bowman D, Couture J, Dimitrov NV, Wolmark N, Wickerham DL, Fisher ER, Margolese R, Robidocex A, Shibotu H, Terz J, Paterson AHG, Lickley HL, Ketner M. A randomized clinical trial evaluating tamoxifen in the treatment of patients with node-negative breast cancer who have estrogen-receptor-positive tumors. *N. Engl. J. Med* 1989;320:479–484. [PubMed: 2644532]
- (2). Osborne CK. Tamoxifen in the treatment of breast cancer. *N. Engl. J. Med* 1998;339:1609–1618. [PubMed: 9828250]
- (3). Hortobagyi GN. Treatment of breast cancer. *N. Engl. J. Med* 1998;339:974–984. [PubMed: 9753714]
- (4). Nayfield SG, Karp JE, Ford LG, Dorr FA, Kramer BS. Potential role of tamoxifen in prevention of breast cancer. *J. Natl. Cancer Inst* 1991;83:1450–1459. [PubMed: 1920492]
- (5). Andersson M, Storm HH, Mouridsen HT. Incidence of new primary cancers after adjuvant tamoxifen therapy and radiotherapy for early breast cancer. *J. Natl. Cancer Inst* 1991;83:1013–1017. [PubMed: 2072407]
- (6). Fisher B, Costantino JP, Redmond CK, Fisher ER, Wickerham DL, Cronin WM. Endometrial cancer in tamoxifen-treated breast cancer patients: findings from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14. *J. Natl. Cancer Inst* 1994;86:527–537. [PubMed: 8133536]
- (7). van Leeuwen FE, Benraadt J, Coebergh JW, Kiemeny LA, Gimbreere CH, Otter R, Schouten LJ, Damhuis RA, Bontenbal M, Diepenhorst FW, van den Belt-Dusebout AW, van Tinteren H. Risk of endometrial cancer after tamoxifen treatment of breast cancer. *Lancet* 1994;343:448–452. [PubMed: 7905955]
- (8). Umemoto A, Monden Y, Komaki K, Suwa M, Kanno Y, Suzuki M, Lin CX, Ueyama Y, Momen MA, Ravindernath A, Shibutani S. Tamoxifen-DNA adducts formed by alpha-acetoxytamoxifen N-oxide. *Chem. Res. Toxicol* 1999;12:1083–1089. [PubMed: 10563834]
- (9). Shibutani S, Suzuki N, Laxmi YR, Schild LJ, Divi RL, Grollman AP, Poirier MC. Identification of tamoxifen-DNA adducts in monkeys treated with tamoxifen. *Cancer Res* 2003;63:4402–4406. [PubMed: 12907611]
- (10). Pathak DN, Pongracz K, Bodell WJ. Activation of 4-hydroxytamoxifen and the tamoxifen derivative metabolite E by uterine peroxidase to form DNA adducts: comparison with DNA adducts formed in the uterus of Sprague-Dawley rats treated with tamoxifen. *Carcinogenesis* 1996;17:1785–1790. [PubMed: 8824496]
- (11). Hemminki K, Rajaniemi H, Lindahl B, Moberger B. Tamoxifen-induced DNA adducts in endometrial samples from breast cancer patients. *Cancer Res* 1996;56:4374–4377. [PubMed: 8813128]
- (12). Shibutani S, Ravindernath A, Suzuki N, Terashima I, Sugarman SM, Grollman AP, Pearl ML. Identification of tamoxifen-DNA adducts in the endometrium of women treated with tamoxifen. *Carcinogenesis* 2000;21:1461–1467. [PubMed: 10910945]
- (13). Shibutani S, Dasaradhi L, Terashima I, Banoglu E, Duffel MW. Alpha-hydroxytamoxifen is a substrate of hydroxysteroid (alcohol) sulfotransferase, resulting in tamoxifen DNA adducts. *Cancer Res* 1998;58:647–653. [PubMed: 9485016]

- (14). Kim SY, Suzuki N, Laxmi YR, McGarrigle BP, Olson JR, Sharma M, Shibutani S. Formation of tamoxifen-DNA adducts in human endometrial explants exposed to alpha-hydroxytamoxifen. *Chem. Res. Toxicol* 2005;18:889–895. [PubMed: 15892583]
- (15). Dowers TS, Qin ZH, Thatcher GR, Bolton JL. Bioactivation of Selective Estrogen Receptor Modulators (SERMs). *Chem. Res. Toxicol* 2006;19:1125–1137. [PubMed: 16978016]
- (16). Dehal SS, Kupfer D. Evidence that the catechol 3,4-Dihydroxytamoxifen is a proximate intermediate to the reactive species binding covalently to proteins. *Cancer Res* 1996;56:1283–1290. [PubMed: 8640815]
- (17). Dinkova-Kostova AT, Holtzclaw WD, Kensler TW. The role of Keap1 in cellular protective responses. *Chem. Res. Toxicol* 2005;18:1779–1791. [PubMed: 16359168]
- (18). Delmas PD, Bjarnason NH, Mitlak BH, Ravoux AC, Shah AS, Huster WJ, Draper M, Christiansen C. Effects of raloxifene on bone mineral density, serum cholesterol concentrations, and uterine endometrium in postmenopausal women. *N. Engl. J. Med* 1997;337:1641–1647. [PubMed: 9385122]
- (19). Phillips C. STAR results: Raloxifene as effective as tamoxifen, better safety profile. *NCI Cancer Bulletin* 2006;3:1–2.
- (20). Dardes RC, Bentrem D, O'Regan RM, Schafer JM, Jordan VC. Effects of the new selective estrogen receptor modulator LY353381.HCl (Arzoxifene) on human endometrial cancer growth in athymic mice. *Clin. Cancer Res* 2001;7:4149–4155. [PubMed: 11751515]
- (21). McMeekin DS, Gordon A, Fowler J, Melemed A, Buller R, Burke T, Bloss J, Sabbatini P. A phase II trial of arzoxifene, a selective estrogen response modulator, in patients with recurrent or advanced endometrial cancer. *Gynecol. Oncol* 2003;90:64–69. [PubMed: 12821343]
- (22). Baselga J, Llombart-Cussac A, Bellet M, Guillem-Porta V, Enas N, Krejcy K, Carrasco E, Kayitalire L, Kuta M, Lluch A, Vodvarka P, Kerbrat P, Namer M, Petruzelka L. Randomized, double-blind, multicenter trial comparing two doses of arzoxifene (LY353381) in hormone-sensitive advanced or metastatic breast cancer patients. *Ann. Oncol* 2003;14:1383–1390. [PubMed: 12954577]
- (23). Yu L, Liu H, Li W, Zhang F, Luckie C, van Breemen RB, Thatcher GR, Bolton JL. Oxidation of raloxifene to quinoids: potential toxic pathways via a diquinone methide and o-quinones. *Chem. Res. Toxicol* 2004;17:879–888. [PubMed: 15257612]
- (24). Liu H, Liu J, van Breemen RB, Thatcher GR, Bolton JL. Bioactivation of the selective estrogen receptor modulator desmethylated arzoxifene to quinoids: 4'-fluoro substitution prevents quinoid formation. *Chem. Res. Toxicol* 2005;18:162–173. [PubMed: 15720120]
- (25). Chen Q, Ngui JS, Doss GA, Wang RW, Cai X, DiNinno FP, Blizzard TA, Hammond ML, Stearns RA, Evans DC, Baillie TA, Tang W. Cytochrome P450 3A4-mediated bioactivation of raloxifene: irreversible enzyme inhibition and thiol adduct formation. *Chem. Res. Toxicol* 2002;15:907–914. [PubMed: 12119000]
- (26). Sasaki M, Kotcherquina L, Dharia A, Fujimoto S, Dahiya R. Cytosine-phosphoguanine methylation of estrogen receptors in endometrial cancer. *Cancer Res* 2001;61:3262–3266. [PubMed: 11309276]
- (27). Liehr JG. Genotoxic effects of estrogens. *Mutat. Res* 1990;238:269–276. [PubMed: 2160607]
- (28). Hayes CL, Spink DC, Spink BC, Cao JQ, Walker NJ, Sutter TR. 17 beta-estradiol hydroxylation catalyzed by human cytochrome P450 1B1. *Proc. Natl. Acad. Sci. U. S. A* 1996;93:9776–9781. [PubMed: 8790407]
- (29). Sharma M, Shubert DE, Lewis J, McGarrigle BP, Bofinger DP, Olson JR. Biotransformation of tamoxifen in a human endometrial explant culture model. *Chem. Biol. Interact* 2003;146:237–249. [PubMed: 14642736]
- (30). Lucas FV, Neufeld HA, Utterback JG, Martin AP, Stotz E. The effect of estrogen on the production of a peroxidase in the rat uterus. *J. Biol. Chem* 1955;214:775–780. [PubMed: 14381415]
- (31). Lyttle CR, DeSombre ER. Uterine peroxidase as a marker for estrogen action. *Proc. Natl. Acad. Sci. U. S. A* 1977;74:3162–3166. [PubMed: 269379]
- (32). Holinka CF, Gurpide E. Peroxidase activity in glands and stroma of human endometrium. *Am. J. Obstet. Gynecol* 1980;138:599–603. [PubMed: 7435524]
- (33). Pongracz K, Pathak DN, Nakamura T, Burlingame AL, Bodell WJ. Activation of the tamoxifen derivative metabolite E to form DNA adducts: comparison with the adducts formed by microsomal activation of tamoxifen. *Cancer Res* 1995;55:3012–3015. [PubMed: 7606720]

- (34). Liu J, Li Q, Yang X, van Breemen RB, Bolton JL, Thatcher GR. Analysis of protein covalent modification by xenobiotics using a covert oxidatively activated tag: raloxifene proof-of-principle study. *Chem. Res. Toxicol* 2005;18:1485–1496. [PubMed: 16167842]
- (35). NIH Guidelines for the Laboratory use of Chemical Carcinogens. U. G. P. O.; Washington DC: 1981. p. 2381–2385.
- (36). Thompson JA, Malkinson AM, Wand MD, Mastovich SL, Mead EW, Schullek KM, Laudenschlager WG. Oxidative metabolism of butylated hydroxytoluene by hepatic and pulmonary microsomes from rats and mice. *Drug Metab. Dispos* 1987;15:833–840. [PubMed: 2893710]
- (37). Hosoya T, Saito T. Comparative studies on estrogen-dependent peroxidases contained in uterine microsomes and fluid of rats and pigs. *J. Biochem. (Tokyo)* 1981;89:203–215. [PubMed: 7194335]
- (38). Storozhenko S, Belles-Boix E, Babiychuk E, Herouart D, Davey SW, Slooten L, Van Montagu M, Inze D, Kushnir S. Gamma-glutamyl transpeptidase in transgenic tobacco plants. Cellular localization, processing, and biochemical properties. *Plant Physiol* 2002;128:1109–1119. [PubMed: 11891265]
- (39). Suojanen JN, Gay RJ, Hilf R. Influence of estrogen on glutathione levels and glutathione-metabolizing enzymes in uteri and R3230AC mammary tumors of rats. *Biochim. Biophys. Acta* 1980;630:485–496. [PubMed: 6104989]
- (40). Hanigan MH, Frierson HF Jr. Immunohistochemical detection of gamma-glutamyl transpeptidase in normal human tissue. *J. Histochem. Cytochem* 1996;44:1101–1108. [PubMed: 8813074]
- (41). Jones RM, Yuan ZX, Lim CK. Tamoxifen metabolism in rat liver microsomes: identification of a dimeric metabolite derived from free radical intermediates by liquid chromatography/mass spectrometry. *Rapid Commun. Mass Spectrom* 1999;13:211–215. [PubMed: 10097399]
- (42). Toader V, Xu X, Nicolescu A, Yu L, Bolton JL, Thatcher GR. Nitrosation, nitration, and autoxidation of the selective estrogen receptor modulator raloxifene by nitric oxide, peroxynitrite, and reactive nitrogen/oxygen species. *Chem. Res. Toxicol* 2003;16:1264–1276. [PubMed: 14565768]
- (43). Smith K, Silvernail NJ, Rodgers KR, Elgren TE, Castro M, Parker RM. Sol-gel encapsulated horseradish peroxidase: a catalytic material for peroxidation. *J. Am. Chem. Soc* 2002;124:4247–4252. [PubMed: 11960453]
- (44). Savitsky PA, Gazaryan IG, Tishkov VI, Lagrimini LM, Ruzgas T, Gorton L. Oxidation of indole-3-acetic acid by dioxygen catalyzed by plant peroxidases: specificity for the enzyme structure. *Biochem. J* 1999;340:579–583. [PubMed: 10359640]
- (45). Hauser MJB, Olsen LF, Bronnikova TV, Schaffer WM. Routes to chaos in the peroxidase-oxidase reaction: period-doubling and period-adding. *J. Phys. Chem. B* 1997;101:5075–5083.
- (46). Adak S, Bandyopadhyay U, Bandyopadhyay D, Banerjee RK. Mechanism of horseradish peroxidase catalyzed epinephrine oxidation: obligatory role of endogenous O_2^- and H_2O_2 . *Biochemistry* 1998;37:16922–16933. [PubMed: 9836585]
- (47). Votyakova TV, Reynolds IJ. Detection of hydrogen peroxide with Amplex Red: interference by NADH and reduced glutathione auto-oxidation. *Arch. Biochem. Biophys* 2004;431:138–144. [PubMed: 15464736]
- (48). Harman LS, Carver DK, Schreiber J, Mason RP. One- and two-electron oxidation of reduced glutathione by peroxidases. *J. Biol. Chem* 1986;261:1642–1648. [PubMed: 3003079]
- (49). Awad HM, Boersma MG, Vervoort J, Rietjens IM. Peroxidase-catalyzed formation of quercetin quinone methide-glutathione adducts. *Arch. Biochem. Biophys* 2000;378:224–233. [PubMed: 10860540]
- (50). Liebler DC. Proteomic approaches to characterize protein modifications: new tools to study the effects of environmental exposures. *Environ. Health Perspect* 2002;110(Suppl 1):3–9. [PubMed: 11834459]
- (51). Poirier MC, Santella RM, Weston A. Carcinogen macromolecular adducts and their measurement. *Carcinogenesis* 2000;21:353–359. [PubMed: 10688855]
- (52). Hummel CW, Geiser AG, Bryant HU, Cohen IR, Dally RD, Fong KC, Frank SA, Hinklin R, Jones SA, Lewis G, McCann DJ, Rudmann DG, Shepherd TA, Tian H, Wallace OB, Wang M, Wang Y, Dodge JA. A selective estrogen receptor modulator designed for the treatment of uterine leiomyoma

with unique tissue specificity for uterus and ovaries in rats. *J. Med. Chem* 2005;48:6772–6775.
[PubMed: 16250633]

Scheme 1.

Formation and Structures of raloxifene and DMA dimers and GSH related conjugates.

Scheme 2.
Synthesis of DMA COATag.

Figure 1.

HPLC-UV chromatogram of the 30 min incubation of SERMs (30 μ M) with rat uterine microsomes (1 mg/mL) in the presence of GSH (500 μ M). A) raloxifene, B) DMA.

Figure 2.

HPLC-UV chromatogram of the 30 min incubation of raloxifene (50 μ M) with HRP (8 unit/mL) in the presence of A) GSH (500 μ M), B) cystine-glycine (500 μ M), C) GSH (500 μ M) and γ -GT (2 unit/mL).

Figure 3.

The effect of CN⁻ (100 μ M), NADPH (1 mM), H₂O₂ (500 μ M) on the oxidation of raloxifene (30 μ M) and DMA (30 μ M) in the presence of GSH (500 μ M) by rat uterine microsomes (1 mg/mL). The assay system is described in Materials and Methods. * p < 0.01, ** p < 0.05, compared to the control, determined by student's t -test. Closed bars, raloxifene; open bars, DMA.

Figure 4.
HPLC-UV chromatogram of the incubation of raloxifene with HRP (8 unit/mL). A) raloxifene (1 μM), B) raloxifene (5 μM), H_2O_2 (10 μM).

Figure 5. HPLC-UV chromatogram of the 30 min incubation of raloxifene (5 μ M) with HRP (8 unit/mL) in the presence of GSH (500 μ M) and different concentrations of hydrogen peroxide. A) without H_2O_2 , B) H_2O_2 (10 μ M), C) H_2O_2 (100 μ M).

Figure 6.

The effect of GSH on the oxidation of raloxifene or DMA (5 μM) by HRP (8 unit/mL). Closed bars, no GSH; open bars, GSH (500 μM).

Figure 7. HPLC-UV chromatogram of the 30 min incubation of raloxifene (5 μ M) with HRP (8 unit/mL) in the presence of GSH (500 μ M) and different concentrations of catalase. A) without catalase, B) catalase (1000 unit/mL), C) catalase (4000 unit/mL)

Figure 8.

Rat liver/uterine microsomes (1.5 mg/mL) was incubated with raloxifene COATag or DMA COATag (30 μ M) in phosphate buffer (pH 7.4), 30 min; a) Rat liver microsomes + raloxifene COATag + NADPH generating system; b) Rat uterine microsomes + raloxifene COATag; c) Rat uterine microsomes + raloxifene COATag + NADPH generating system; d) Rat uterine microsomes + raloxifene COATag + H_2O_2 ; e) Rat liver microsomes + DMA COATag + NADPH generating system; f) Rat uterine microsomes + DMA COATag; g) Rat uterine microsomes + DMA COATag + NADPH generating system; h) Rat uterine microsomes + DMA COATag + H_2O_2 . Concentrations of NADPH or H_2O_2 used in the incubation system were 1 mM.

Mechanism 1 **Mechanism 2** **GSH Oxidation** **Scheme 3.**

Mechanism of oxidation of raloxifene by peroxidase in the presence or absence of GSH.