Bioactivation of the Selective Estrogen Receptor Modulator Acolbifene to Quinone Methides

Ju Liu, Hong Liu, Richard B. van Breemen, Gregory R. J. Thatcher, and Judy L. Bolton*

Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, 833 South Wood Street, M/C 781, Chicago, Illinois 60612-7231

Received August 11, 2004

Although approved for the treatment of hormone-dependent breast cancer as well as for the prevention of breast cancer in high-risk women, the selective estrogen receptor modulator (SERM) tamoxifen has been associated with an increased risk of endometrial cancer in women. With an understanding of the potential carcinogenic mechanisms of these compounds, SERMs could in principle be designed or selected for use that avoids these problems. Acolbifene (EM-652) is a fourth-generation SERM and the active form of the ester prodrug EM-800. As a pure antagonist of breast tumor development and growth, acolbifene does not stimulate endometrial tissue. However, acolbifene was found in this investigation to form two kinds of quinone methides, either through chemical or through enzymatic oxidation. One was a classical acolbifene quinone methide, which was formed by oxidation at the C-17 methyl group, and the other was a diquinone methide involving the oxidation of two phenol groups. The half-life of the classical quinone methide was determined to be 32 ± 0.4 s at physiological pH and temperature. The quinone methides reacted with glutathione (GSH) to form five mono-GSH conjugates and five di-GSH conjugates. The majority of GSH conjugates resulted from reaction of the classical acolbifene quinone methide with GSH. Incubations of acolbifene with GSH and either tyrosinase or human and rat liver microsomes also produced acolbifene quinone methide−GSH conjugates. In addition to reaction with GSH, the classical acolbifene quinone methide was also shown to react with deoxynucleosides. One of the major deoxynucleoside adducts was identified as the deoxyadenosine adduct resulting from reaction of the classical acolbifene quinone methide with the exocyclic amino group of adenine. Acolbifene could also induce DNA damage in the S30 breast cancer cell line. These data imply that the classical electrophilic acolbifene quinone methide might contribute to the potential toxicity of acolbifene.

Introduction

Although estrogen replacement therapy reduces the severity of menopausal symptoms (*1*−*3*), prolonged exposure to estrogens increases the risk of developing certain hormone-dependent cancers. An alternative is to use compounds that act as estrogen receptor agonists in certain tissues and as estrogen receptor antagonists in others. These compounds are known as selective estrogen receptor modulators (SERMs)[1] and include such well-known compounds as tamoxifen and raloxifene (Figure 1) (*4*, *5*).

The nonsteroidal antiestrogen tamoxifen is a triphenylethylene that has been widely used since the 1970s in the treatment of all stages of hormone-dependent breast cancer (*6*). Although recent large scale clinical trials of tamoxifen as a chemopreventive agent in women considered at high risk of developing breast cancer demonstrated protection in 45% of the subjects (*7*), there is evidence that tamoxifen has been associated with developing uterine cancer and an increase in the inci-

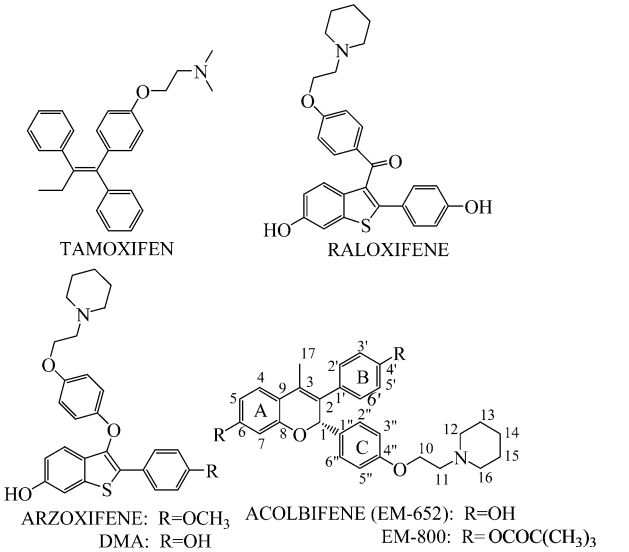


Figure 1. Structures of tamoxifen, raloxifene, arzoxifene, and acolbifene.

* To whom correspondence should be addressed. Phone: (312) 996-5280. Fax: (312) 996−7107. E-mail: Judy.Bolton@UIC.edu.

[1] Abbreviations: AC-dA, acolbifene deoxyadenosine adduct; AC-dC, acolbifene deoxycytosine adduct; AC-dG, acolbifene deoxyguanosine adduct; AC-T, acolbifene thymidine adduct; AC-SG, acolbifene mono-GSH conjugate; AC-di-SG, acolbifene di-GSH conjugate; AC-QM, classical acolbifene quinone methide; CID, collision-induced dissociation; DMA, desmethylated arzoxifene; DMA-di-QM, DMA diquinone methide; ER, estrogen receptor; LC-MS-MS, liquid chromatography−tandem mass spectrometry; MRM, multiple reaction monitoring; 4-OHTAM, 4-hydroxytamoxifen; 4-OHTAM-QM, 4-hydroxytamoxifen quinone methide; RAL-di-QM, raloxifene diquinone methide; SERM, selective estrogen receptor modulator.

10.1021/tx0497752 CCC: $30.25
Published on Web 01/15/2005

dence of endometrial cancer (*8*−*10*). The molecular mechanism(s) involved in the associated risks of tamoxifen still remains elusive. One potential mechanism might involve biotransformation of tamoxifen to electrophilic metabolites including a carbocation (*11*), an *o*-quinone (*12*), and a quinone methide (*13*), which could modify critical cellular macromolecules (*14*−*16*).

Raloxifene has been approved by the Food and Drug Administration for prevention of osteoporosis in postmenopausal women (*17*). Preclinical studies with raloxifene showed an antiestrogenic effect on rat mammary tumor growth that was equal to that of tamoxifen. However, raloxifene demonstrated significantly less estrogenic effects on the uterus than tamoxifen (*18*). The STAR trial (Studies on Tamoxifen and Raloxifene) is in progress and is designed to examine which of these SERMs is most effective in the prevention of breast cancer in high risk women (*19*). However, it should be noted that recent in vitro studies have shown that raloxifene can also be metabolized to electrophilic quinoids (*20*, *21*).

Based on the success of raloxifene, structural analogues have been developed. One such SERM, arzoxifene is a benzothiophene analogue of raloxifene that has a more favorable therapeutic and safety profile than raloxifene in preclinical studies (*18*). Arzoxifene was more effective than raloxifene with respect to bone metabolism and in inhibiting the growth of ER-positive breast cancer cells in vitro (*19*). However, like raloxifene and tamoxifen, DMA (a desmethylated metabolite of arzoxifene) was also efficiently converted to electrophilic metabolites including a diquinone methide and *o*-quinones, which formed corresponding GSH conjugates. Formation of such reactive metabolites might contribute to the potential toxicity of arzoxifene (*22*).

In the present study, the potential for another SERM, acolbifene, to form electrophilic compounds was investigated. Acolbifene is an active benzopyrene derivative of EM-800, a fourth-generation SERM, and is the most potent antiestrogen in terms of inhibition of both ERα and ERβ (*23*). EM-800 is an extremely potent inhibitor of the proliferation of breast and endometrial cancer cells in culture (*24*, *25*) and has also been shown to prevent the growth of estrogen-stimulated tumor xenografts in athymic mice (*26*). Furthermore, EM-800 also prevents bone loss and cardiovascular disease in rats (*27*). Although no toxic effects of acolbifene have yet been reported, we have found that, like tamoxifen and other SERMs, acolbifene can be oxidized either chemically or enzymatically to acolbifene quinone methides. These quinone methides were also shown to form adducts with GSH or deoxynucleosides. In addition, acolbifene was shown to be cytotoxic to breast cancer cells in culture and cause DNA damage. Together, these data suggest that formation of quinone methides might be an important cytotoxic pathway for acolbifene.

Materials and Methods

Materials and Instrumentation. Caution: *Acolbifene quinone methides were handled in accordance with the NIH Guidelines for the Laboratory Use of Chemical Carcinogens* (*28*). All solvents and chemicals were purchased from Aldrich Chemical (Milwaukee, WI), Fisher Scientific (Itasca, IL), or Sigma (St. Louis, MO) unless stated otherwise. Acolbifene was synthesized as described previously (*29*). The same instruments and conditions for obtaining the NMR and mass spectra reported previously were used in this investigation (*20*, *22*). The comet assay was performed as described in the previous manuscript in this issue (*22*).

HPLC. Three general methods were used to analyze and separate the various metabolites and conjugates. All the retention times reported in the text were obtained using method A unless stated otherwise. UV absorbance was measured at 318 nm. For method A, analytical HPLC analysis was performed using a 4.6 mm × 250 mm Ultrasphere C_{18} column (Beckman, Fullerton, CA) at a flow rate of 1.0 mL/min. The mobile phase consisted of 20% methanol in 0.1% formic acid, which was increased to 35% methanol over 5 min. After the phase was held at 35% methanol from 5 to 15 min, the methanol content was increased to 50% over 25 min and from 50% to 60% over 5 min. Finally, the methanol content was increased to 95% over 2 min before returning to the initial solvent composition. During LC-MS-MS, the flow was split so that only 200 μL/min entered the mass spectrometer. For method B, for the analysis of di-GSH conjugates, the mobile phase composition remained identical to method A, but the gradient was changed as follows: 5 min isocratic 20% methanol, 5 min linear gradient from 20% to 30% methanol, 20 min isocratic 30% methanol, 15 min linear gradient from 30% to 60% methanol, and finally a 2 min gradient from 60% to 95%. For method C, semipreparative HPLC was carried out using a 10 mm × 250 mm Ultrasphere C_{18} column at a flow rate of 3.0 mL/min. The initial mobile phase consisted of 30% methanol in 0.1% formic acid, followed by a 6 min linear gradient to 35% methanol, then a 29 min gradient from 35% to 50% methanol, 50−54% methanol over 15 min, and finally a 5 min gradient from 54% to 95% methanol.

Preparation of Acolbifene Quinone Methides. A mixture of acolbifene (10 mg, 0.022 mmol) in anhydrous acetonitrile (9 mL) and freshly prepared silver oxide (1 g) was stirred for 5 min at room temperature. The yellow supernatant containing the classical acolbifene quinone methide and acolbifene diquinone methide was removed and analyzed immediately.

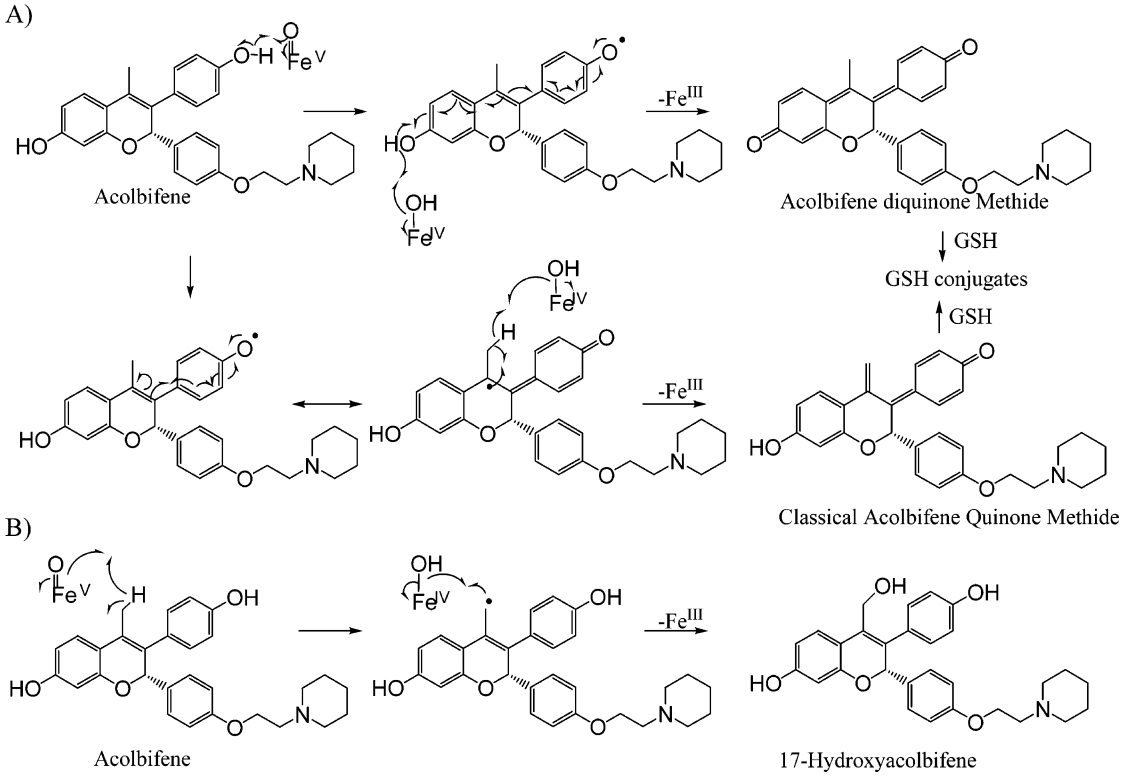
Kinetic Experiments. An aliquot (0.1 mL) of the acetonitrile preparation of acolbifene quinone methides (0.2 mM) was mixed with 0.9 mL of 50 mM K_2HPO_4 buffer (pH 7.4) at 37 °C, and the disappearance of the acolbifene quinone methides was monitored by measuring the decrease in UV absorbance at 430 nm (5 s/scan) using a Hewlett-Packard 8452A diode array spectrophotometer.

Reaction of Acolbifene Quinone Methides with GSH. A mixture of 2 mM acolbifene quinone methides in 1 mL of acetonitrile and 20 mM GSH in 9 mL of phosphate buffer (50 μM, pH 7.4) was stirred at room temperature for 5 min. Perchloric acid (50 μL/mL) was added, and the solution was concentrated under a stream of N_2. The reaction mixtures were centrifuged at 176 062 × *g* for 6 min, and the supernatant was removed, loaded onto PrepSep C_{18} solid-phase extraction cartridges (0.5 mL), washed with water (3 × 1 mL), and eluted with methanol (3 × 1 mL). The methanol eluate was concentrated to approximately 1 mL. Aliquots (20 μL) of the mixture were analyzed by using LC-MS-MS and HPLC method A. The remaining solution was purified using semipreparative HPLC.

Oxidation of Acolbifene by Tyrosinase. A mixture of acolbifene (0.1 mM), tyrosinase (0.1 mg/mL), and GSH (1 mM) in 50 mM phosphate buffer (pH 7.4, 1.0 mL total volume) was incubated for 30 min at 37 °C. Reactions were terminated by chilling in an ice bath for 1 min followed by the addition of perchloric acid (50 μL/mL). Control incubations were carried out without tyrosinase or GSH. The reaction mixtures were loaded onto solid-phase extraction cartridges and eluted with methanol as described above. The methanol eluate was concentrated to approximately 100 μL, and 50 μL aliquots were analyzed using LC-MS-MS.

Oxidation of Acolbifene by Human and Rat Liver Microsomes. A solution containing the substrate (0.1 mM), rat liver microsomes (1 nmol of P450/mL), GSH (1 mM), and a NADPH-generating system (including 1.0 mM $NADP^+$, 5.0 mM $MgCl_2$, 5.0 mM isocitric acid, and 0.2 unit/mL isocitrate dehy-

Scheme 1. Mechanism of Formation of (A) Acolbifene−GSH Conjugates and (B) 17-Hydroxyacolbifene



drogenase) in 50 mM phosphate buffer (pH 7.4, 1 mL total volume) was incubated for 30 min at 37 °C (*30*). For control incubations, either $NADP^+$ or GSH was omitted. The reactions were terminated by chilling in an ice bath for 1 min followed by the addition of perchloric acid (50 μL/mL). The proteins were removed by centrifugation at 176 062 × *g* for 6 min. Excess acolbifene in the supernatant was removed with *tert*-butyl methyl ether, and the aqueous solution was extracted, concentrated, and analyzed as described above. Incubations of human liver microsomes (pooled from 15 individuals, In Vitro Technologies, Baltimore, MD) were carried out using similar procedures except that the concentration of P450 was 0.34 nmol/mL. In addition, the incubations of the substrate, GSH, and rat liver microsomes in $H_2{}^{18}O$ (100 μL) were also performed using similar procedures. The proteins were removed using ultrafiltration through a 5K molecular weight cutoff regenerated cellulose filter (Millipore, Bedford, MA). A 50 μL aliquot was analyzed directly using LC-MS-MS and HPLC method A.

Reaction of Acolbifene Quinone Methides with Deoxynucleosides. A solution of acolbifene quinone methides (0.8 mM) in 2.5 mL of acetonitrile was combined with each of the four deoxynucleosides (20 mM) in 10 mL of phosphate buffer (pH 7.4). The solution was incubated for 4 h at 37 °C, extracted using solid-phase extraction cartridges, eluted with methanol, and concentrated to a final volume of 1 mL under a stream of N_2. An aliquot (50 μL) was analyzed using LC-MS-MS and HPLC method A. The most abundant deoxyadenosine adduct (AC-dA-1) was collected using semipreparative HPLC for NMR analysis.

Cytotoxicity Studies of Acolbifene in S30 Cells. The trypan blue exclusion assay was conducted to determine cell viability (*31*). The S30 cell line, a stable ERα transfectant of MDA-MB-231 cells, was a generous gift from V. C. Jordan (Northwestern University, Chicago, IL). The S30 cells were maintained in phenol-red-free MEME (minimal essential media with Earle's salts) supplemented with 1% penicillin−streptomycin−fungizome (PSF), 6 μg/L insulin, 1% glutamax (Gibco-BRL, Grand Island, NY), 5% charcoal-dextran-treated fetal bovine serum, 500 μg/mL G418, and 5% CO_2 at 37 °C. The cells were grown for 24 h to maintain logarithmic growth and then treated with various concentrations of test compounds or DMSO in fresh medium for 18 h. Compounds were freshly dissolved in DMSO, and the final DMSO concentration was 0.2%. After treatment, floating cells were collected, attached cells were first trypsinized, and then floating and attached cells were combined. All cells were harvested by centrifugation at 2 000 rpm for 5 min. Trypan blue stain (0.1 mL of 0.4%) was added to each cell suspension at a total volume of 0.6 mL. The cell suspension was transferred to a hemacytometer, and the number of cells was counted under a microscope. The dead cells were stained blue and viable cells excluded the stain. The LC_{50} values were obtained by regression and linear estimation analysis. The data represent the means ± SD of three determinations.

Results

Kinetic Studies of Acolbifene Quinone Methides. Two types of acolbifene quinone methides were formed from acolbifene using silver oxide as the chemical oxidizing agent. One was a classical acolbifene quinone methide, which was formed by oxidation at the C-17 methyl group, and the other was a diquinone methide involving the oxidation of two phenol groups (Scheme 1). The UV spectra of the reaction mixture showed a strong absorbance at 430 nm (in acetonitrile), which was absent in the starting material and is a characteristic of classical quinone methides (*32*, *33*). Unlike the diquinone methides formed from raloxifene and DMA (*20*, *22*), which have λ_{max} exceeding 490 nm, the classical acolbifene quinone methide should absorb at a shorter wavelength compared to that of acolbifene diquinone methide due to the less extensive π conjugated system. As a result, the absorbance at λ_{max} 430 nm is mainly due to the acolbifene classical quinone methide and not the acolbifene diquinone methide. The positive ion electrospray MS of the acolbifene quinone methides showed a protonated molecule of *m/z* 456, which was two mass units less than that of acolbifene and consistent with the formation of

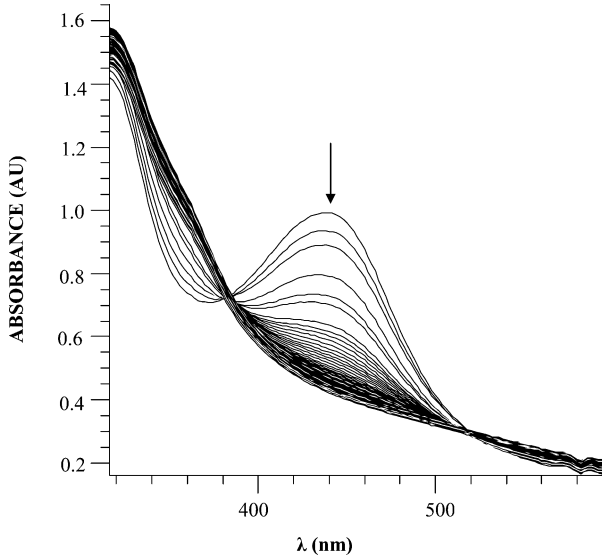


Figure 2. The decomposition of acolbifene quinone methides (0.2 mM) in 50 mM phosphate buffer (pH 7.4, 37 °C) was monitored at 430 nm. UV spectra were recorded continuously from 200 to 600 nm at 5 s/scan.

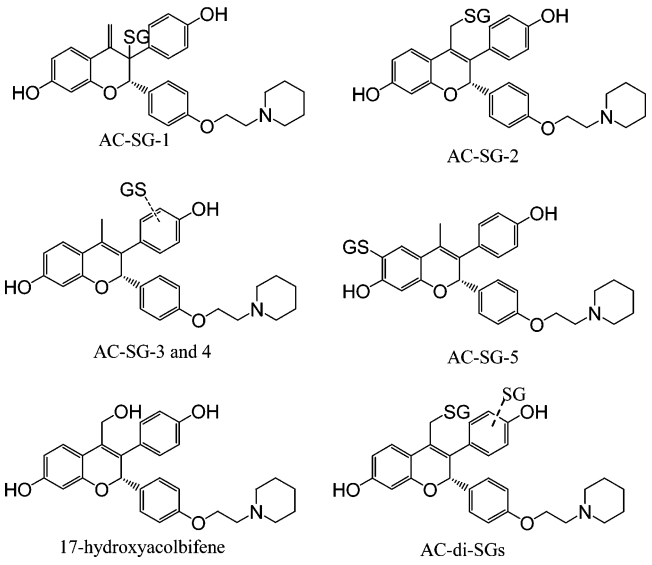


Figure 3. Structures of acolbifene−GSH conjugates and 17-hydroxyacolbifene.

acolbifene quinone methides. At physiological pH and temperature, the half-life of the classical acolbifene quinone methide was determined to be 32 ± 0.4 s (Figure 2).

Reactivity of Acolbifene Quinone Methides with GSH. The chemically oxidized acolbifene quinone methides reacted with GSH to give five mono-GSH conjugates and five di-GSH conjugates (Figures 3 and 4). The mono-GSH conjugates were arbitrarily assigned as AC-SG-1 (peak 1), AC-SG-2 (peak 2), AC-SG-3 (peak 4), AC-SG-4 (peak 5), and AC-SG-5 (peak 6) in Figure 4. The retention times for the AC-SG-1 and AC-SG-2 were considerably different from those of AC-SG-3, AC-SG-4, and AC-SG-5 (21.8, 29.4, 45.8, 46.4, and 47.1 min, respectively, Figure 4). The major products (AC-SG-1 and AC-SG-2) were formed from reaction of GSH with the classical acolbifene quinone methide, and the other products (AC-SG-3−5) resulted from reaction with the acolbifene diquinone methide. MS-MS with CID of the protonated molecules of AC-SG-1 to AC-SG-5 at *m/z* 763 produced abundant

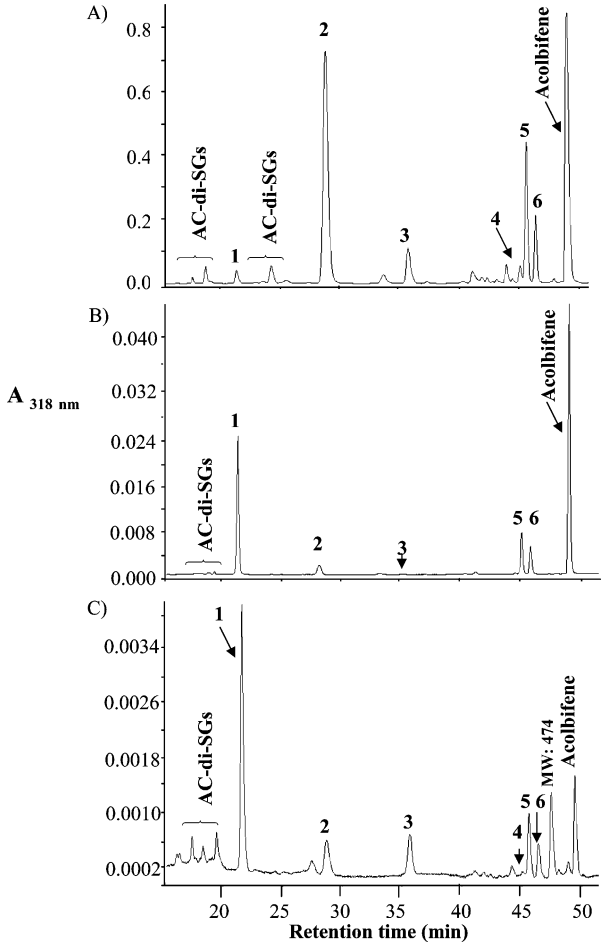


Figure 4. HPLC chromatograms of acolbifene−GSH conjugates formed by incubation of GSH with (A) chemically oxidized acolbifene, (B) acolbifene and tyrosinase, and (C) acolbifene and rat liver microsomes: (**1**) AC-SG-1; (**2**) AC-SG-2; (**3**) 17-hydroxyacolbifene; (**4**) AC-SG-3; (**5**) AC-SG-4; (**6**) AC-SG-5.

product ions of *m/z* 634 corresponding to the loss of a γ-glutamyl group (Figures 5 and 6). The fragment ion of *m/z* 112 corresponded to a vinylpiperidine ion. The loss of water from the protonated molecule of *m/z* 763 produced the fragment ion at *m/z* 745. Cleavage on either side of the sulfur produced ions of 490 or 456; however, the ion of *m/z* 456 was not observed in the tandem mass spectra of AC-SG-3−5 (Figure 6). Based on the differences in HPLC retention times, tandem mass spectra, and NMR (see below), GSH attacked the classical acolbifene quinone methide at either C-17 or C-2 forming AC-SG-1 and -2 (Figure 3). For the other three conjugates, GSH added to ring carbons of the diquinone methide, which would have reduced the probability of forming the ion of *m/z* 456 since that would require the energetically unfavorable cleavage of the bond between S and an aromatic ring.

The structures of these five mono-GSH conjugates were determined with ^{1}H and distortionless enhancement by polarization transfer (DEPT)-NMR by comparison to acolbifene. In the ^{1}H NMR (Table 1) of AC-SG-1 (Figure 3), all the protons except the three protons in C-17 methyl group were similar to the acolbifene protons. Two vinyl protons (at 5.00 and 5.25 ppm, respectively) replaced the signal corresponding to the three protons in the C-17 methyl group. In the DEPT-NMR, a methylene carbon at approximately 110 ppm was observed as opposed to

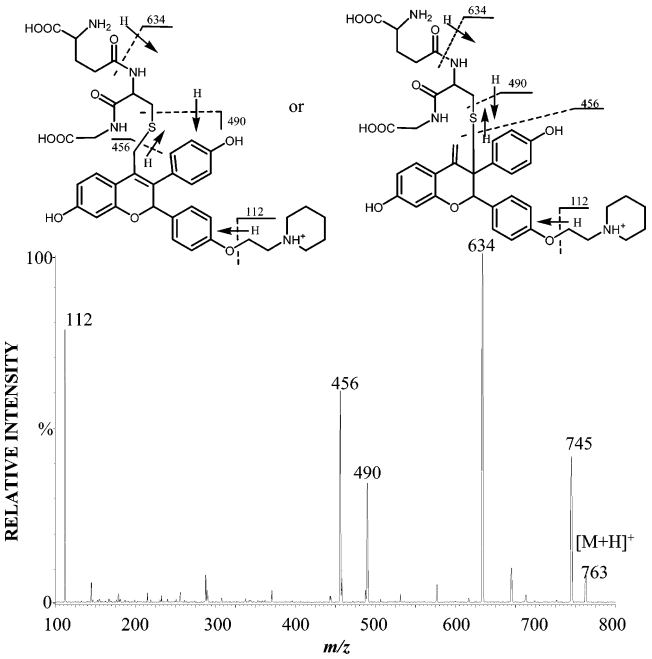


Figure 5. Positive ion electrospray product ion tandem mass spectrum with CID of the protonated molecule of AC-SG-1 or AC-SG-2 at *m/z* 763.

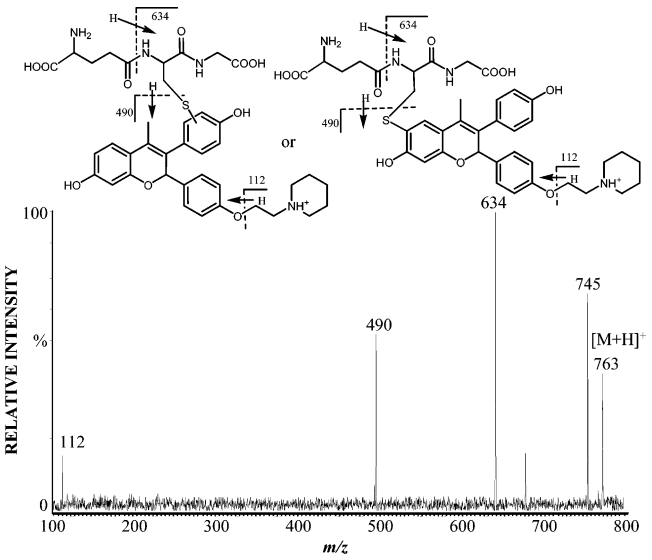


Figure 6. Positive ion electrospray product ion tandem mass spectrum with CID of the protonated molecule of AC-SG-3 at *m/z* 763. The corresponding tandem mass spectra of AC-SG-4 and AC-SG-5 were essentially identical.

the C-17 methyl carbon signal. Therefore, it was concluded that GSH added to the C-2 position forming a double bond between C-3 and C-17.

In the ^{1}H NMR spectrum of AC-SG-2 (Table 1, Figure 3), the three protons in the C-17 methyl group were replaced by two protons in a methylene group at ~3.56 ppm. In the DEPT-NMR, the C-17 methyl carbon signal (~15 ppm) was replaced by a methylene carbon signal (~21 ppm). As a result, it was determined that GSH must add in the C-17 position for AC-SG-2.

The disappearance of one proton in the B ring (Figure 1) suggested that GSH substitution had occurred at the B ring for both AC-SG-3 and AC-SG-4 (Figure 3). H-5 was absent allowing the assignment of AC-SG-5 (Figure 3). The structure of AC-SG-1 and -2 confirmed our hypothesis that the C-17 methyl group participated in the formation of the classical acolbifene quinone methide (Scheme 1). Furthermore, the formation of AC-SG-4, AC-SG-5, and AC-SG-6 suggested that a small amount of the acolbifene diquinone methide coexisted with the classical acolbifene quinone methide during the oxidation of acolbifene (Scheme 1).

In addition to mono-GSH conjugates, acolbifene quinone methides formed di-GSH conjugates that eluted at 17.2, 18.1, 19.2, 24.9, and 34.3 min, using HPLC method B. All five di-GSH conjugates formed singly and doubly protonated molecules of *m/z* 1068 and 534.5, respectively, during positive ion electrospray mass spectrometry and deprotonated molecules of *m/z* 1066 during negative ion electrospray (data not shown). The fragmentation patterns of all five di-GSH conjugates were similar during MS-MS with CID, so the conjugates could not be distinguished. However, fragment ions provided ions that were characteristic of GSH conjugates (*34*−*36*). For example, MS-MS with CID of the deprotonated molecules of *m/z* 1066 produced fragment ions of *m/z* 937, 919, 808, corresponding to loss of one γ-glutamyl group, a γ-glutamyl group plus water, and elimination of two γ-glutamyl moieties, respectively. In addition, elimination of a glutathione group, loss of a γ-glutamyl group from one glutathione moiety, and cleavage of the thioether moiety from the other glutathione from the deprotonated molecule produced the product ions of *m/z* 761 and 664, respectively. The instability of the di-GSH conjugates precluded the isolation of sufficient material for characterization using NMR. We suggest that the presence of the electron-donating sulfur substituent facilitated oxidation of the aromatic ring resulting in reaction with solvent nucleophiles and dimerization reactions.

Production of 17-Hydroxyacolbifene. 17-Hydroxyacolbifene was formed from acolbifene during reaction of chemically generated acolbifene quinone methides with GSH and in enzymatic incubations (either tyrosinase or human and rat liver microsomes) with acolbifene in the presence of GSH. The retention time was 36.4 min. During positive ion MS-MS analysis, a protonated molecule was detected at *m/z* 474, which indicated that acolbifene had been monoxygenated. MS-MS product ion scanning of this protonated molecule at *m/z* 474 generated an abundant fragment ion at *m/z* 112 corresponding to a vinylpiperidine ion. In addition, structural characterization was obtained using ^{1}H NMR (Table 1). Signals to all protons except the three at the C-17 methyl group could be correlated to those of the acolbifene, which indicated that the hydroxylation occurred at the C-17 methyl group. The two protons of C-17 shifted downfield to 3.41 ppm due to the deshielding effect of the hydroxyl group. For the incubations of acolbifene with rat liver microsomes in $H_2{}^{18}O$ either in the presence of GSH or without GSH, the protonated molecule of 17-hydroxyacolbifene at *m/z* 474 without ^{18}O incorporation was observed (data not shown).

Oxidation of Acolbifene. Since P450 3A isozymes are known to metabolize polyphenols to quinoids (*37*), we used microsomes isolated from rats that had been treated with dexamethasone to induce the P450 3A isozymes. For validation of this rat model, pooled human liver microsomes were also used in separate experiments. Tyrosinase is also often used as a catalyst for the oxidation of polyphenols and was incubated with acolbifene to investigate its oxidation. As indicated by the LC-MS-MS

Table 1. ^{1}H NMR of Acolbifene and Its Conjugates in d_4-MeOH/D_2O, 2:1

	chemical shifts (ppm), multiplicity, *J* (Hz), and integral								
compounds	H2″6″	H4	H2′6′	H3″5″	H3′5′	H5	H7	H1	H17
acolbifene	7.30, d *J* = 8.6 2H	7.18, d *J* = 8.4 1H	7.13, d *J* = 8.5 2H	6.92, d *J* = 8.6 2H	6.78, d *J* = 8.5 2H	6.40, dd *J* = 2.4,8.4 1H	6.15, d *J* = 2.3 1H	6.00, s 1H	2.09, s 3H
AC-SG-1	6.79, d *J* = 8.3 2H	7.70, d *J* = 8.8 1H	6.68, d *J* = 8.8 2H	6.66, d *J* = 8.8 2H	6.46, d *J* = 8.6 2H	6.50, dd *J* = 8.8 1H	6.23, d *J* = 2.1 1H	5.98, s 1H	5.25, s(α); 5.00, s(*β*) 1H, 1H
AC-SG-2	7.19, d *J* = 8.6 2H	7.25, d *J* = 8.5 1H	7.04, d *J* = 8.5 2H	6.75, d *J* = 8.6 2H	6.64, d *J* = 8.5 2H	6.28, dd *J* = 2.3,8.5 1H	6.00, d *J* = 2.3 1H	5.70, s 1H	3.56, s 2H
17-hydroxyacolbifene	7.24, d *J* = 8.7 2H	7.33, d *J* = 8.5 1H	7.08, d *J* = 8.6 2H	6.81, d *J* = 8.7 2H	6.71, d *J* = 8.6 2H	6.38, dd *J* = 2.5,8.5 1H	6.10, d *J* = 2.5 1H	5.83, s 1H	3.41, s 2H
AC-SG-3	7.15, t 3H	7.05, d 1H	6.85, d 1H		6.68, t 3H	6.25, dd 1H	6.03, d 1H	5.71, s 1H	2.00, s 3H
AC-SG-4	7.18, t 3H	7.16, d 1H	7.07, d 1H	7.01, d 1H	6.78, t 2H	6.39, dd 1H	6.11, d 1H	5.82, s 1H	1.97, s 3H
AC-SG-5	7.11, d *J* = 8.6 2H	7.26, s 1H	6.88, d *J* = 8.5 2H	6.70, d *J* = 8.6 2H	6.61, d *J* = 8.5 2H		6.12, s 1H	5.70, s 1H	1.95, s 3H

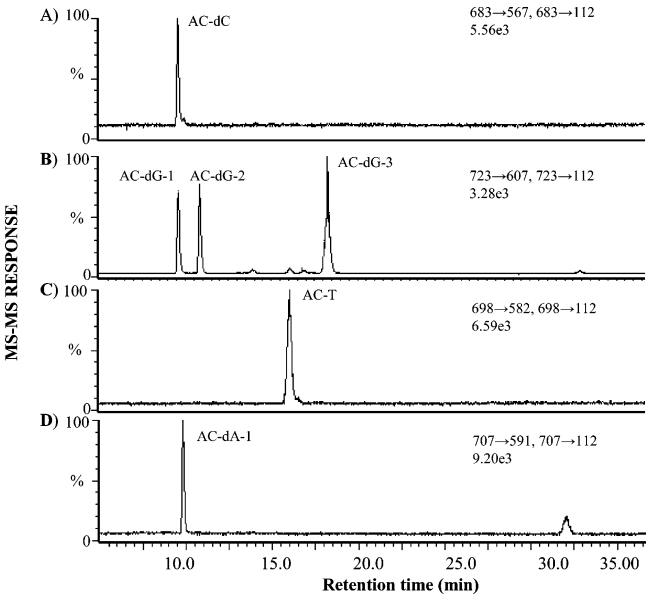
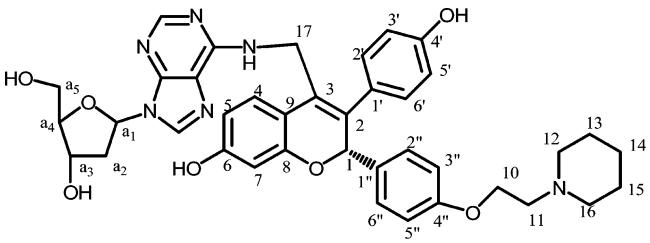


Figure 7. LC-MS-MS MRM chromatograms of acolbifene quinone methide adducts with deoxynucleosides: (A) AC-dC; (B) AC-dG; (C) AC-T; (D) AC-dA.

Table 2. ^{1}H NMR of AC-dA-1 in DMSO-d_6



chemical shifts (ppm), multiplicity, *J* (Hz), and integral							
H2″6″	H2′6′	H4	H3″5″	H3′5′	H5	a_1H	H7
7.24, d *J* = 8.6 2H	7.10, d *J* = 8.1 2H	7.07, d *J* = 8.5 1H	6.83, d *J* = 8.6 2H	6.68, d *J* = 8.4 2H	6.23, dd *J* = 8.5 1H	6.18, t *J* = 6.6 1H	6.13,d *J* = 2.2 1H

chemical shifts (ppm), multiplicity, *J* (Hz), and integral							
H1	N*H*	a_3-O*H*	a_5-O*H*	17-CH_2	a_3H	H10	a_4H
6.00, s 1H	5.55, d 1H	5.27, d 1H	4.89, b 1H	4.77, m 2H	4.34, m 1H	4.02, t *J* = 5.5 2H	3.83, q *J* = 3.1 1H

chemical shifts (ppm), multiplicity, *J* (Hz), and integral							
$a_{5\alpha}H$	$a_{5\beta}H$	H11	$a_{2\alpha}$H	H12,16	$a_{2\beta}$H	H13,15	H14
3.51, m 1H	3.49, m 1H	2.73 2H	2.57, m 1H	2.50, b 4H	2.26, m 1H	1.48, b 4H	1.37, b 2H

analyses of these incubations, rat liver microsomes as well as tyrosinase catalyzed oxidation of acolbifene to quinone methides, which then reacted with GSH in vitro to form mono- and di-GSH conjugates (Figure 4). Experiments with human liver microsomes gave similar results (data not shown). Among these three enzyme systems, incubation with tyrosinase produced the greater yield of quinone methides and their GSH conjugates. Although similar GSH conjugates were observed in enzymatic or chemical oxidation systems containing GSH, AC-SG-1 predominated in the enzymatic system incubations, whereas AC-SG-2 was more abundant in the chemically oxidized acolbifene system.

Reaction of Acolbifene Quinone Methides with Deoxynucleosides. The classical acolbifene quinone methide reacted with each of the four deoxynucleosides to form adducts. As shown in the LC-MS-MS chromatograms (Figure 7), two deoxyadenosine adducts, one thymidine adduct, three deoxyguanosine adducts, and one deoxycytosine adduct were detected. During LC-MS-MS with CID, each protonated acolbifene−deoxynucleoside adduct fragmented to eliminate deoxyribosyl, $[MH - 116]^+$. In addition, ions of *m/z* 112 were detected corresponding to vinylpiperidine. Because of low yields, only the most abundant deoxyadenosine adduct, AC-dA-1, which formed from the classical acolbifene quinone methide, was analyzed using NMR, and the structure and ^{1}H NMR assignments are shown in Table 2.

In the correlation spectroscopy (COSY)-NMR of AC-dA-1, two protons at 4.77 ppm were attributed to C-17 protons due to the coupling with the NH proton ($\delta = 5.55$ ppm). All the other protons in the aromatic rings correlated similarly to the acolbifene protons. As a result, the dA adduct resulted from reaction between the adenine exocyclic amino group and the C-17 position on the classical quinone methide.

Cytotoxicity Studies and "Comet Assay" in S30 Cells. Following incubation with test compounds, the trypan blue exclusion assay and "comet assay" (more than 80% viability) were conducted to determine cell

Table 3. Cytotoxicity of Antiestrogens in the S30 Breast Cancer Cell Line and Reactivity of Quinone Methides[a]

substrate	LC_{50} (μM)	metabolite	$t_{1/2}$
4-OHTAM[b]	19 ± 2	4-OHTAM-QM[c]	174 ± 41 min
raloxifene[d]	55 ± 1	RAL-di-QM[d]	< 1 s
DMA	27 ± 1	DMA-di-QM[e]	15 ± 0.4 s
acolbifene	22 ± 3	AC-QM	32 ± 0.4 s

[a] Cells (10^5 cells/mL) were incubated with various concentrations of tested substrates for 18 h. Cell viability was determined by trypan blue exclusion. Half-lives were determined by monitoring of the disappearance of quinone methides in phosphate buffer (pH 7.4, 37 °C). Results represent the means ± SD of three determinations. [b] Reference *45*. [c] Reference *13*. [d] Reference *20*. [e] Reference *22*.

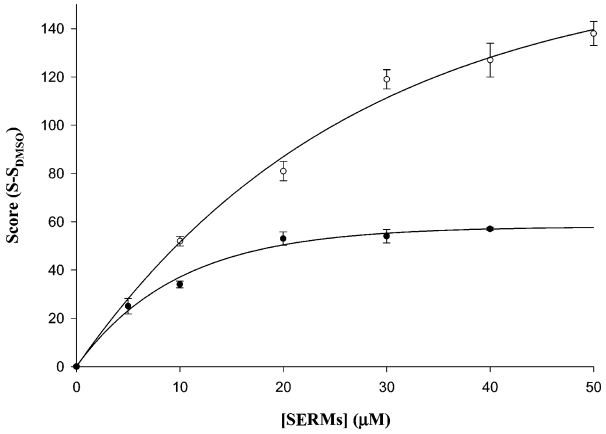


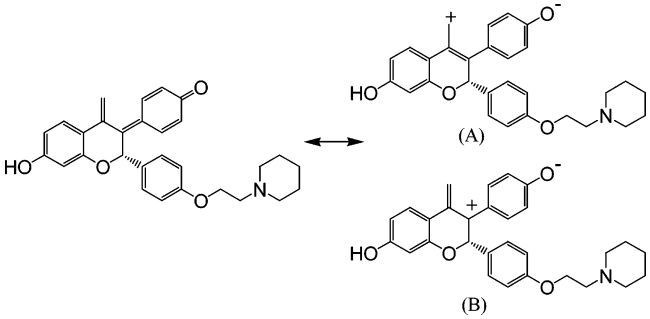
Figure 8. Induction of DNA damage by acolbifene (○) and raloxifene (●) in the S30 cell line. Cells (2×10^5 cells/mL) were treated with vehicle or different concentrations of acolbifene or raloxifene for 90 min.

viability and DNA damage in the breast cancer cell line S30 (ER^+). The S30 cell line is an estrogen receptor positive cell line obtained from the MDA-MB-231 cell line through stable transfection with ERα. Acolbifene was shown to be more toxic than raloxifene and had equivalent potency to DMA in this cell line (Table 3). The "comet assay" is an alkaline single cell gel electrophoresis assay that is widely used to study DNA damage (*38*). These data indicated that acolbifene induced dose-dependent DNA damage and induced more DNA damage than raloxifene in the S30 cell line (Figure 8).

Discussion

The formation of quinoids is involved in the toxicity of both estrogens and antiestrogens through alkylation of cellular macromolecules, generation of reactive oxygen species, or both (*14*, *15*, *39*). Previous studies have shown that tamoxifen, raloxifene, and DMA can be metabolized to quinones and quinone methides that react with cellular nucleophiles such as GSH (*13*, *20*, *22*). In this study, the acolbifene quinone methides were generated chemically and enzymatically. The half-life of the classical acolbifene quinone methide (32 ± 0.4 s) was considerably shorter than that of 4-hydroxytamoxifen quinone methide (174 min) but longer than that of the raloxifene diquinone methide ($t_{1/2}$ < 1 s, Table 3). This indicates that the classical acolbifene quinone methide is an electrophile of intermediate stability. Thompson et al. (*40*) reported that quinone methides with half-lives in the range of 10 s to 10 min had time to diffuse away from the site of formation and react with vital cellular macromolecules. These quinone methides of intermediate reactivity are more likely to react with cellular nucleophiles and cause toxicity than more reactive compounds that will react immediately with another quinone methide, solvent, O_2, or the enzyme responsible for quinone methide formation leading to suicide inhibition as observed with raloxifene (*21*). As a result, acolbifene has the potential to be more toxic than raloxifene by forming the more stable classical acolbifene quinone methide. The formation of 17-hydroxyacolbifene without ^{18}O incorporation during the incubations of acolbifene with rat liver microsomes in $H_2{}^{18}O$ suggests that 17-hydroxyacolbifene was formed via a P450-mediated hydroxylation mechanism instead of reaction of the classical acolbifene quinone methide with water from which ^{18}O incorporation would be expected (Scheme 1). The classical acolbifene quinone methide may be selective enough to attack sulfur or nitrogen nucleophiles or both on cellular macromolecules rather than react with a poor nucleophile like water, which suggests that formation of the classical acolbifene quinone methide could result in toxicity in vivo.

Scheme 2. Resonance Structures of the Classical Acolbifene Quinone Methide



Acolbifene quinone methides reacted with GSH forming mono- and di-GSH conjugates. In particular, AC-SG-1 and AC-SG-2 resulting from the attack of GSH with the classical quinone methide were the most abundant conjugates. Quinone methides have been described as resonance-stabilized carbocations (*41*, *42*) because of the important contribution of the charged aromatic resonance form. The electronic distribution of any particular quinone methide should depend on the ability of substituent groups and solvent molecules to support charge separation. It is possible that the resonance structure A (Scheme 2) represents the major contribution to the overall resonance hybrid during the silver oxide oxidation of acolbifene. Since C-17 is less sterically hindered, this is the more favorable site of reaction with GSH resulting in formation of AC-SG-2 as the major conjugate. In the enzymatic experiments, resonance structure B may contribute more than the other resonance structures due to the extended conjugation of the alkene group with the aromatic π system and the stabilization of charge by water. Therefore, in this situation, GSH adds at the C-2 position to form AC-SG-1 as the most abundant product. Furthermore, only one diastereoisomer of AC-SG-1 resulted from the attack of GSH at the prochiral benzyl carbon of the acolbifene quinone methide. This is in contrast to the reactions of the hepatocarcinogen safrole quinone methide and eugenol quinone methide with GSH, in which two diastereoisomers were observed (*33*, *43*, *44*). Therefore, GSH must attack C-2 from the

opposite side of the benzyl piperidine group due to steric effects.

In contrast to studies with DMA and raloxifene, the classical acolbifene quinone methide reacted with each of the four deoxynucleosides to form adducts. LC-MS-MS analysis of incubations of the DMA diquinone methide with deoxynucleosides only gave one adduct with deoxyguanosine (*22*). Although several experiments were done to detect adducts formed from raloxifene diquinone methide with deoxynucleosides, none were observed (unpublished results). It is possible that the classical acolbifene quinone methide is more reactive with deoxynucleosides as compared to the DMA and raloxifene diquinone methides, which might contribute to the potential genotoxicity of acolbifene in vivo.

There is a noticeable difference in toxicity in the S30 cell line among the three SERMs (4-hydroxytamoxifen, DMA, and acolbifene) and raloxifene (Table 3). The relatively longer half-lives of the other three quinone methides compared to that of raloxifene (Table 3) may account for these differences in toxicity. The raloxifene diquinone methide might be too reactive to cause extensive cellular damage. DNA damage detected by the "comet assay" in this cell line showed that both acolbifene and DMA induced more DNA damage than raloxifene. The stability and reactivity of the quinone methide intermediates might also account for this difference. Moreover, the induction of DNA damage suggests that raloxifene, acolbifene, and DMA might have potential to damage cellular DNA through the generation of the reactive oxygen species.

Even though both the classical acolbifene quinone methide and acolbifene diquinone methide were formed during the oxidation of acolbifene, the classical acolbifene quinone methide was the major metabolite, which is in contrast to DMA and raloxifene, from which only diquinone methides were formed. The formation of the classical acolbifene quinone methide might represent the major toxic pathway for acolbifene by forming more GSH and deoxynucleoside adducts as compared to the diquinone methide.

In conclusion, we have found that acolbifene can be oxidized to acolbifene quinone methides either chemically or enzymatically and these quinone methides can react with GSH or deoxynucleosides under physiological conditions. However, the classical acolbifene quinone methide contributes more than the acolbifene diquinone methide in forming adducts either with GSH or with deoxynucleosides. In addition, the cytotoxic potency of acolbifene in the S30 human breast cancer cell line and the induction of DNA damage in this cell line suggest that acolbifene might be more toxic than some other SERMs such as raloxifene. These data suggest that the classical acolbifene quinone methides might contribute to the potential cytotoxicity, genotoxicity, or both of acolbifene through depletion of GSH and alkylation of cellular macromolecules. The results of this investigation and similar studies of other SERMs will be useful in the design and development of SERMs that maintain efficacy as antiestrogens without the potential problems associated with genetoxic and cytotoxic oxidation products.

Acknowledgment. This work was supported by NIH Grant CA 79870. We thank Dr. Minsun Chang and Mr. Yan Li for assistance with the "comet assay". We also thank Dr. Connie Luckie for constructive suggestions.

References

(1) Worley, R. J. (1981) Age, estrogen, and bone density. *Clin. Obstet. Gynecol. 24*, 203−218.

(2) Stampfer, M. J., Colditz, G. A., and Willett, W. C. (1990) Menopause and heart disease. A review. *Ann. N. Y. Acad. Sci. 592*, 193−203; discussion 257−162.

(3) Barrett-Connor, E., and Grady, D. (1998) Hormone replacement therapy, heart disease, and other considerations. *Annu. Rev. Public Health 19*, 55−72.

(4) Meegan, M. J., and Lloyd, D. G. (2003) Advances in the science of estrogen receptor modulation. *Curr. Med. Chem. 10*, 181−210.

(5) Jordan, V. C. (2003) Antiestrogens and selective estrogen receptor modulators as multifunctional medicines. 2. Clinical considerations and new agents. *J. Med. Chem. 46*, 1081−1111.

(6) Furr, B. J., and Jordan, V. C. (1984) The pharmacology and clinical uses of tamoxifen. *Pharmacol. Ther. 25*, 127−205.

(7) Fisher, B., Costantino, J. P., Wickerham, D. L., Redmond, C. K., Kavanah, M., Cronin, W. M., Vogel, V., Robidoux, A., Dimitrov, N., Atkins, J., Daly, M., Wieand, S., Tan-Chiu, E., Ford, L., and Wolmark, N. (1998) Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *J. Natl. Cancer Inst. 90*, 1371−1388.

(8) King, C. M. (1995) Tamoxifen and the induction of cancer. *Carcinogenesis 16*, 1449−1454.

(9) Phillips, D. H. (2001) Understanding the genotoxicity of tamoxifen? *Carcinogenesis 22*, 839−849.

(10) van Leeuwen, F. E., Benraadt, J., Coebergh, J. W., Kiemeney, L. A., Gimbráere, C. H., Otter, R., Schouten, L. J., Damhuis, R. A., Bontenbal, M., Diepenhorst, F. W., et al. (1994) Risk of endometrial cancer after tamoxifen treatment of breast cancer. *Lancet 343*, 448−452.

(11) Umemoto, A., Komaki, K., Monden, Y., Suwa, M., Kanno, Y., Kitagawa, M., Suzuki, M., Lin, C. X., Ueyama, Y., Momen, M. A., Ravindernath, A., and Shibutani, S. (2001) Identification and quantification of tamoxifen-DNA adducts in the liver of rats and mice. *Chem. Res. Toxicol. 14*, 1006−1013.

(12) Zhang, F., Fan, P. W., Liu, X., Shen, L., van Breeman, R. B., and Bolton, J. L. (2000) Synthesis and reactivity of a potential carcinogenic metabolite of tamoxifen: 3,4-dihydroxytamoxifen-*o*-quinone. *Chem. Res. Toxicol. 13*, 53−62.

(13) Fan, P. W., Zhang, F., and Bolton, J. L. (2000) 4-Hydroxylated metabolites of the antiestrogens tamoxifen and toremifene are metabolized to unusually stable quinone methides. *Chem. Res. Toxicol. 13*, 45−52.

(14) Bolton, J. L. (2002) Quinoids, quinoid radicals, and phenoxyl radicals formed from estrogens and antiestrogens. *Toxicology 177*, 55−65.

(15) Bolton, J. L., Pisha, E., Zhang, F., and Qiu, S. (1998) Role of quinoids in estrogen carcinogenesis. *Chem. Res. Toxicol. 11*, 1113−1127.

(16) Kim, S. Y., Suzuki, N., Laxmi, Y. R., and Shibutani, S. (2004) Genotoxic mechanism of tamoxifen in developing endometrial cancer. *Drug Metab. Rev. 36*, 199−218.

(17) Delmas, P. D., Bjarnason, N. H., Mitlak, B. H., Ravoux, A. C., Shah, A. S., Huster, W. J., Draper, M., and Christiansen, C. (1997) Effects of raloxifene on bone mineral density, serum cholesterol concentrations, and uterine endometrium in postmenopausal women. *N. Engl. J. Med. 337*, 1641−1647.

(18) Conzen, S. D. (2003) Current status of selective estrogen receptor modulators (SERMs). *Cancer J. 9*, 4−14.

(19) Sato, M., Turner, C. H., Wang, T., Adrian, M. D., Rowley, E., and Bryant, H. U. (1998) LY353381.HCl: a novel raloxifene analogue with improved SERM potency and efficacy in vivo. *J. Pharmacol. Exp. Ther. 287*, 1−7.

(20) Yu, L., Liu, H., Li, W., Zhang, F., Luckie, C., Van Breemen, R. B., Thatcher, G. R., and Bolton, J. L. (2004) Oxidation of Raloxifene to Quinoids: Potential Toxic Pathways via a Diquinone Methide and *o*-Quinones. *Chem. Res. Toxicol. 17*, 879−888.

(21) Chen, Q., Ngui, J. S., Doss, G. A., Wang, R. W., Cai, X., DiNinno, F. P., Blizzard, T. A., Hammond, M. L., Stearns, R. A., Evans, D. C., Baillie, T. A., and Tang, W. (2002) Cytochrome P450 3A4-mediated bioactivation of raloxifene: irreversible enzyme inhibition and thiol adduct formation. *Chem. Res. Toxicol. 15*, 907−914.

(22) Liu, H., Yu, L., Liu, J., van Breeman, R. B., Thatcher, G. R. J., and Bolton, J. L. (2004) Bioactivaton of the selective estrogen receptor modulator desmethylated arzoxifene to quinoids: 4′-

fluoro substitution prevents quinoid formation. *Chem. Res. Toxicol.* **2005**, *18*, 162−173.
(23) Gauthier, S., Caron, B., Cloutier, J., Dory, Y. L., Favre, A., Larouche, D., Mailhot, J., Ouellet, C., Schwerdtfeger, A., Leblanc, G., Martel, C., Simard, J., Mâerand, Y., Bâelanger, A., Labrie, C., and Labrie, F. (1997) (*S*)-(+)-4-[7-(2,2-Dimethyl-1-oxopropoxy)-4-methyl-2-[4-[2-(1-piperidinyl)-ethoxy]phenyl]-2*H*-1-benzopyran-3-yl]-phenyl 2,2-dimethylpropanoate (EM-800): a highly potent, specific, and orally active nonsteroidal antiestrogen. *J. Med. Chem. 40*, 2117−2122.
(24) Simard, J., Labrie, C., Bâelanger, A., Gauthier, S., Singh, S. M., Mâerand, Y., and Labrie, F. (1997) Characterization of the effects of the novel nonsteroidal antiestrogen EM-800 on basal and estrogen-induced proliferation of T-47D, ZR-75-1 and MCF-7 human breast cancer cells in vitro. *Int. J. Cancer 73*, 104−112.
(25) Simard, J., Sanchez, R., Poirier, D., Gauthier, S., Singh, S. M., Merand, Y., Belanger, A., Labrie, C., and Labrie, F. (1997) Blockade of the stimulatory effect of estrogens, OH-tamoxifen, OH-toremifene, droloxifene, and raloxifene on alkaline phosphatase activity by the antiestrogen EM-800 in human endometrial adenocarcinoma Ishikawa cells. *Cancer Res. 57*, 3494−3497.
(26) Couillard, S., Gutman, M., Labrie, C., Bâelanger, A., Candas, B., and Labrie, F. (1998) Comparison of the effects of the antiestrogens EM-800 and tamoxifen on the growth of human breast ZR-75−1 cancer xenografts in nude mice. *Cancer Res. 58*, 60−64.
(27) Luo, S., Labrie, C., Bâelanger, A., and Labrie, F. (1997) Effect of dehydroepiandrosterone on bone mass, serum lipids, and dimethylbenz[*a*]anthracene-induced mammary carcinoma in the rat. *Endocrinology 138*, 3387−3394.
(28) *NIH Guidelines for the Laboratory Use of Chemical Carcinogens* (1981), NIH Publication, U.S. Government Printing Office, Washington, DC, pp 81−2385.
(29) Draper, R. W., Iyer, R. V., Lu, Y., and Vater, E. J. Enantioselective synthesis, U.S. Patent 6,262,270 B1, 2001.
(30) Iverson, S. L., Shen, L., Anlar, N., and Bolton, J. L. (1996) Bioactivation of estrone and its catechol metabolites to quinoid-glutathione conjugates in rat liver microsomes. *Chem. Res. Toxicol. 9*, 492−499.
(31) Freshney, R. (1987) *Culture of Animal cells: A Manual of Basic Techniques*, p 117, Alan R. Liss Inc., New York.
(32) Filar, L. J., and Winstein, S. (1960) Preparation and behavior of simple quinone methides. *Tetrahedron Lett. 25*, 9−16.
(33) Bolton, J. L., Comeau, E., and Vukomanovic, V. (1995) The influence of 4-alkyl substituents on the formation and reactivity of 2-methoxy-quinone methides: evidence that extended pi-conjugation dramatically stabilizes the quinone methide formed from eugenol. *Chem.-Biol. Interact. 95*, 279−290.
(34) Kassahun, K., Davis, M., Hu, P., Martin, B., and Baillie, T. (1997) Biotransformation of the naturally occurring isothiocyanate sulforaphane in the rat: identification of phase I metabolites and glutathione conjugates. *Chem. Res. Toxicol. 10*, 1228−1233.
(35) Ramanathan, R., Cao, K., Cavalieri, E., and Gross, M. L. (1998) Mass spectrometric methods for distinguishing structural isomers of glutathione conjugates of estrone and estradiol. *J. Am. Soc. Mass Spectrom. 9*, 612−619.
(36) Cao, K., Stack, D. E., Ramanathan, R., Gross, M. L., Rogan, E. G., and Cavalieri, E. L. (1998) Synthesis and structure elucidation of estrogen quinones conjugated with cysteine, *N*-acetylcysteine, and glutathione. *Chem. Res. Toxicol. 11*, 909−916.
(37) Koop, D. R., Laethem, C. L., and Schnier, G. G. (1989) Identification of ethanol-inducible P450 isozyme 3a (P450IIE1) as a benzene and phenol hydroxylase. *Toxicol. Appl. Pharmacol. 98*, 278−288.
(38) Olive, P. L., Banâath, J. P., and Durand, R. E. (1990) Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the "comet" assay. *Radiat. Res. 122*, 86−94.
(39) Bolton, J. L., Trush, M. A., Penning, T. M., Dryhurst, G., and Monks, T. J. (2000) Role of quinones in toxicology. *Chem. Res. Toxicol. 13*, 135−160.
(40) Thompson, D. C., Perera, K., Krol, E. S., and Bolton, J. L. (1995) *o*-Methoxy-4-alkylphenols that form quinone methides of intermediate reactivity are the most toxic in rat liver slices. *Chem. Res. Toxicol. 8*, 323−327.
(41) Richard, J. P., Ameyes, T. L., Bei, L., and Stubblefield, V. (1990) The effect of β-fluorine substituents on the rate and equilibrium constants for the reactions of α-substituted 4-methoxybenzyl carbocations and on the reactivity of a simple quinone methide. *J. Am. Chem. Soc. 112*, 9513−9519.
(42) Hulbert, P. B., and Grover, P. L. (1983) Chemical rearrangement of phenol-epoxide metabolites of polycyclic aromatic hydrocarbons to quinone-methides. *Biochem. Biophys. Res. Commun. 117*, 129−134.
(43) Bolton, J. L., Acay, N. M., and Vukomanovic, V. (1994) Evidence that 4-allyl-*o*-quinones spontaneously rearrange to their more electrophilic quinone methides: potential bioactivation mechanism for the hepatocarcinogen safrole. *Chem. Res. Toxicol. 7*, 443−450.
(44) Thompson, D., Constantin-Teodosiu, D., Norbeck, K., Svensson, B., and Moldâeus, P. (1989) Metabolic activation of eugenol by myeloperoxidase and polymorphonuclear leukocytes. *Chem. Res. Toxicol. 2*, 186−192.
(45) Yao, D., Zhang, F., Yu, L., Yang, Y., van Breemen, R. B., and Bolton, J. L. (2001) Synthesis and reactivity of potential toxic metabolites of tamoxifen analogues: droloxifene and toremifene *o*-quinones. *Chem. Res. Toxicol. 14*, 1643−1653.

TX0497752