Bioactivation of the Selective Estrogen Receptor Modulator Desmethylated Arzoxifene to Quinoids: 4′-Fluoro Substitution Prevents Quinoid Formation

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Although selective estrogen receptor modulators (SERMs) are useful in the treatment and prevention of breast cancer, the SERM tamoxifen has been associated with an increased risk of endometrial cancer possibly due to metabolism to electrophilic quinoids. Another SERM, arzoxifene is currently in clinical trials for the treatment of breast cancer, and since it has similar structural characteristics to tamoxifen, it also has the potential to form quinoids. In the current study, the active form of arzoxifene in vivo, desmethylated arzoxifene (DMA), was synthesized and chemically or enzymatically oxidized to DMA diquinone methide. The half-life of DMA diquinone methide at physiological pH and temperature was approximately 15 s. Reaction of DMA diquinone methide with glutathione (GSH) gave four mono-GSH conjugates, two di-GSH conjugates, and one tri-GSH conjugate. In incubations of DMA with GSH and either rat or human liver microsomes, DMA o-quinone–GSH conjugates were detected in addition to DMA diquinone methide–GSH conjugates. A DMA diquinone methide–deoxyguanosine adduct was detected following the incubation of DMA diquinone methide with deoxyribonucleosides. In preliminary studies with a human breast cancer cell line, DMA induced dose-dependent DNA damage and was more effective at causing DNA damage than raloxifene. These results suggest that DMA can be metabolized to electrophilic/redox-active quinoids, which have the potential to cause toxicity in vivo. A new fluorinated derivative unable to form a diquinone methide, 4′-F-DMA, was synthesized. 4′-F-DMA showed similar estrogen receptor (ER) binding affinity as compared to DMA. The antiestrogenic activity as measured by inhibition of estradiol-mediated induction of alkaline phosphatase activity in Ishikawa cells showed 10-fold lower activity for 4′-F-DMA compared to DMA; however, the antiestrogenic activity was comparable to raloxifene. In microsomal incubations of 4′-F-DMA in the presence of GSH, no GSH adducts were detected. These data suggest that 4′-F-DMA might be a promising SERM with similar activity to DMA and raloxifene and less toxicity.

Introduction

Breast cancer is one of the most common cancers in the Western Hemisphere and the second most common cause of cancer deaths in women (1). Tamoxifen (Figure 1) has been the mainstay of hormonal therapy for women with hormone-dependent breast cancer (2, 3) since the 1970s. Although tamoxifen has been very successful in the treatment of breast cancer, it is associated with an increased risk of endometrial hyperplasia and cancer (4–6). The most widely recognized mechanism for the carcinogenicity of tamoxifen is estrogen receptor (ER)1-mediated hormonal activity (7). The proliferative activity of estrogens and SERMs at the ER may promote carcinogenesis; however, an alternative initiation mechanism could involve cytochrome P450-mediated metabolic activation of these compounds to form either redox-active or electrophilic metabolites, which act as chemical carcinogens by modification of cellular macromolecules (8–10). For example, tamoxifen can be metabolized to at least three different metabolites, including α-hydroxytamoxifen (11), 4-hydroxytamoxifen (12), and 3,4-dihydroxytamoxifen (13), all of which are further metabolized to electrophilic species.

Due to the potential side effects related to tamoxifen, considerable attention has been paid to development of less toxic SERMs. A benzothiophene SERM, raloxifene (Figure 1), is in clinical use for the prevention and treatment of postmenopausal osteoporosis (14) and is currently in clinical trials for chemoprevention of breast cancer (STAR trial; Study of Tamoxifen and Raloxifene) (15). Owing to low efficacy in women with advanced breast cancer, raloxifene has not been pursued as a treatment (16). Furthermore, raloxifene also has the potential to cause blood clots and unfavorable vasomotor symptoms. Raloxifene has been reported to undergo oxidation in rat liver microsomes to yield a diquinone methide and o-quinone, which are electrophilic, reactive intermediates that form conjugates with glutathione.

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1 Abbreviations: CID, collision-induced dissociation; DMA, desmethylated arzoxifene; DMA-SG, glutathionyl-desmethylated arzoxifene; ER, estrogen receptor; LC-MS-MS, liquid chromatography–tandem mass spectrometry; MEME, minimal essential media with Earle’s salts; SERM, selective estrogen receptor modulator.
adducts were detected. 4.Both arzoxifene and its active metabolite desmethylated arzoxifene (DMA) bind to the ER with high affinity and inhibit estrogen-dependent growth of MCF-7 breast cancer cells. The antiproliferation potency of arzoxifene against MCF-7 cells is 3000–25 000-fold higher than that of tamoxifen (23). In addition, unlike tamoxifen, arzoxifene acts as an estrogen antagonist in the uterus (24). In a phase II trial of arzoxifene in advanced endometrial cancer, arzoxifene showed high antitumor activity (25). The use of arzoxifene versus tamoxifen as a first-line therapy in metastatic breast cancer is currently being studied in a multicenter European phase III trial (26). Since arzoxifene and DMA are close structural analogues of raloxifene and DMA are close structural analogues of raloxifene, it is crucial to determine whether DMA can be metabolized in an analogous manner to form electrophiles that have the potential to act as chemical carcinogens.

In the current study, DMA was synthesized and oxidized either chemically or enzymatically to a diquinone methide and then reacted with GSH to give mono-, di-, and tri-GSH conjugates. In microsomal incubations, DMA o-quinone–GSH conjugates were detected in addition to DMA diquinone methide–GSH conjugates. We also compared the relative ability of DMA and raloxifene to induce DNA damage in breast cancer cell lines using the comet assay. The data showed that DMA could be metabolized to electrophilic, redox-active quinoids, which have the potential to cause toxicity in vivo. A new compound 4′-F-DMA was designed to reduce toxicity while still retaining biological activity. The results showed that 4′-F-DMA binds to ERα and ERβ with affinities similar to those of DMA and has antiestrogenic activity comparable to raloxifene. In microsomal incubations with 4′-F-DMA in the presence of GSH, no GSH adducts were detected. 4′-F-DMA induced much less DNA damage as compared to DMA using comet assay. As a result, 4′-F-DMA demonstrates that SERMs may be designed with attenuated toxicity that maintain ER binding and ER modulating activity.

Materials and Methods

Caution: DMA diquinone methide was handled in accordance with the NIH Guidelines for the Laboratory Use of Chemical Carcinogens (27).

Materials. All solvents and reagents were purchased from either Aldrich Chemical (Milwaukee, WI), Fisher Scientific (Itasca, IL), or Sigma (St. Louis, MO) unless stated otherwise. DMA was prepared by a modification of a published procedure (28).

Instrumentation. 1H NMR and 13C NMR spectra were obtained with Bruker DRX500, AVANCE360, or AVANCE300 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 either an Agilent (Palo Alto, CA) ion trap mass spectrometer equipped with a model 1100 HPLC system and electrospray ionization or a Micromass (Manchester, U.K.) Quattro II triple quadrupole mass spectrometer equipped with a Waters (Milford, MA) 2690 HPLC system. Collision-induced dissociation (CID) was carried out using a range of collision energies from 25 to 70 eV and an argon collision gas pressure of 1.2 μbar. High-resolution electrospray mass spectra were obtained using a Micromass Q-Tof-2 hybrid mass spectrometer.

HPLC Methods. Three methods were used to analyze and separate metabolites and conjugates. For method A, analytical HPLC analysis was performed using a Beckman 4.6 mm × 250 mm UltraspHERE C18 column with UV absorbance detection at 316 nm. The mobile phase consisted of a linear gradient from acetonitrile to water that contains 10% methanol and 0.1% formic acid at 1 mL/min as follows: 10–30% acetonitrile over 20 min, 10 min gradient from 30% to 70% acetonitrile, and then 70–90% acetonitrile over 5 min. For method B, semipreparative HPLC was used to isolate conjugates for additional characterization. Separation was carried out using an UltraspHERE C18 column (10 mm × 250 mm) and a mobile phase consisting of a 50 min linear gradient from 5% to 30% acetonitrile with a cosolvent of water containing 10% methanol and 0.1% formic acid at a flow rate of 3 mL/min. For method C, analytical HPLC analysis of DMA diquinone methide deoxynucleoside adducts was performed using an UltraspHERE C18 column (4.6 mm × 25 mm) with UV absorbance detection at 316 nm. The mobile phase consisted of a 50 min linear gradient from 10% to 70% methanol in water that contained 0.5% ammonium acetate (pH 3.5).

Synthesis of Desmethylated Arzoxifene (DMA) (Scheme 1).

1. 6-Methoxy-2-(4-methoxyphenyl)-3-bromobenz(6,3)-thiophene (2). N-Bromoacetamide (2.92 g, 21 mmol) in 20 mL of ethanol was added dropwise to a solution of 6-methoxy-2-(4-methoxyphenyl)benzo[b]thiophene (1) (5.0 g, 18.5 mmol) in 750 mL of dichloromethane and 40 mL of ethanol at room temperature. After the solution was stirred for 1 h, the solvent was removed in vacuo. 6-Methoxy-2-(4-methoxyphenyl)-3-bromobenz(6,3)-thiophene (2) (6.2 g, 96%) was recrystallized from hexane/ethyl acetate (1:1, v/v).

2. 6-Methoxy-2-(4-methoxyphenyl)-3-bromobenz(6,3)-thiophene S-Oxide (3). Trifluoacetic acid (25 mL) was added dropwise to a solution of 2 (5.0 g, 14.3 mmol) in 25 mL of anhydrous dichloromethane. After the mixture was stirred for 5 min, H2O2 (2.0 mL, 14.3 mmol, 30% aqueous solution) was added dropwise, and the resulting mixture was stirred for 2 h at room temperature. Sodium bisulfite (0.7 g) was added to this solution followed by 10 mL of water. The mixture was stirred vigorously for 15 min and then concentrated in vacuo. The residue was partitioned between CH2Cl2 and saturated aqueous
NaHCO₃ solution (100 mL each). The layers were separated, and the organic layer was washed consecutively with portions of water, saturated NaHCO₃, and water. The organic layer was dried over anhydrous Na₂SO₄ and concentrated in vacuo to a solid that was purified by flash chromatography (SiO₂, hexane/ethyl acetate (6:1, v/v)) to obtain 4.6 g (81%) of 6-hydroxy-3-[4-(2-piperidinyl)ethoxy]phenol. This material was converted to the hydrochloride salt by treatment with diethyl ether·HCl in ethyl acetate (91% yield).

**Scheme 1. Synthesis of Desmethylated Arzoxifene (DMA)**

- 1. 1-(4-Fluorophenyl)-2-(3-methoxyphenyl)sulfanyl)ethanone (5 g, 18 mmol) was added to this solution at a rate such that the temperature did not exceed 25 °C, and the reaction mixture was stirred at 0 °C for 2.5 h. The reaction was quenched by the slow addition of 2.5 mL of 2.0 M NaOH. The resulting precipitate was collected and dried over anhydrous Na₂SO₄. After concentration in vacuo to an oil, the residue was purified by flash chromatography (SiO₂, chloroform/methanol (5:1, v/v)) to yield 1.4 g (90%) of 6-hydroxy-3-[4-(2-piperidinyl)ethoxy]phenol.

- 2. Methoxy-3-[4-(2-piperidinyl)ethoxy]phenyoxy (6). LiAlH₄ (0.17 g, 4.46 mmol) was added in small portions to a solution of 4-[2-(1-piperidinyl)ethoxy]phenol (113 mg, 0.51 mmol, 60% dispersion in mineral oil) was added to this solution followed by stirring at 0 °C for 1 h. The mixture was stirred for 1 h, ethyl acetate and water were added, and the organic layer was washed several times with water and then dried over anhydrous Na₂SO₄. The residue was purified by flash chromatography (SiO₂, hexane/chloroform (6:1, v/v)) to give 2.5 g of 6-hydroxy-3-[4-(2-piperidinyl)ethoxy]phenol. This material was converted to the hydrochloride salt by treatment with diethyl ether·HCl in ethyl acetate (91% yield).

- 3. 4-[2-(1-Piperidinyl)ethoxy] Phenol (4). 4-[2-(1-Piperidinyl)ethoxy] phenol (4) was prepared as described previously (28).

**Synthesis of 4′-Fluoro-desmethylated Arzoxifene (4′-FDMA)** (Scheme 2). 1. 1-(4-Fluorophenyl)-2-(3-methoxyphenylsulfanyl)ethanone (8). 3-Methoxybenzenethiol (1 g, 7.1 mmol) was added in one portion to a freshly prepared solution of 7.5 mL of ethanol, 3 mL of water, and 470 mg of KOH (8.4 mmol). The solution was cooled to 5–10 °C. A solution of 2-bromo-1-(4-fluorophenyl)ethanone (1.54 g, 7.1 mmol) in 2.5 mL of EtOAc was added to this solution at a rate such that the temperature did not exceed 25 °C, and the reaction mixture was allowed to stir overnight at room temperature. The solvents were removed under reduced pressure, and the residue was partitioned between water and ethyl acetate. The aqueous layer was isolated and extracted several times with ethyl acetate, and the combined extracts were washed with consecutive portions of 10% HCl, water, saturated NaHCO₃, and water again before being dried over anhydrous Na₂SO₄. After concentration in vacuo to an oil, the crude product was purified by flash chromatography (SiO₂, hexane/ethyl acetate/methanol (5:5:1, v/v/v)) to yield 200 mg (81%) of 5 as a white solid.

**Scheme 1.** Synthesis of Desmethylated Arzoxifene (DMA)²

**Scheme 2.** 1. 1-(4-Fluorophenyl)-2-(3-methoxyphenylsulfanyl)ethanone (8). 3-Methoxybenzenethiol (1 g, 7.1 mmol) was placed into the beaker; 1-(4-fluorophenyl)-2-(3-methoxyphenylsulfanyl)ethanone (5 g, 18 mmol) was added to this solution at a rate such that the temperature did not exceed 25 °C, and the reaction mixture was stirred at 0 °C for 1 h. The mixture was stirred for 1 h, ethyl acetate and water were added, and the organic layer was washed several times with water and then dried over anhydrous Na₂SO₄. The residue was purified by flash chromatography (SiO₂, hexane/chloroform (6:1, v/v)) to give 2.5 g of 9. **H NMR (360 MHz, DMSO-d₆) δ 8.33 (s, 3H), 7.00 (dd, J = 2.4, 8.8 Hz, 1H).**
NaHCO₃, and water, and then dried over anhydrous Na₂SO₄. The residue was titrated with hexane/ether and filtered to give 3.0 g (89%) of 6-hydroxy-3-thiophene hydrochloride (12).

4. 6-Methoxy-2-(4-fluorophenyl)-3-bromobenzothiophene S-oxide (11). Trifluoroacetic acid (13 mL) was added to a solution of 12 (2.4 g, 5.7 mmol) in 25 mL of anhydrous DMF at room temperature. After the mixture was stirred for 15 min and then concentrated in vacuo; the residue was titrated with diethyl ether and filtered to yield 2.1 g (85%) of 11.

5. 6-Methoxy-3-[4-2-(1-piperidinyl)ethoxy]benzo[b]thiophene S-oxide (12). NaH (237 mg, 9.9 mmol, 60% dispersion in mineral oil) was added to a solution of 4 (1.31 g, 59 mmol) in 25 mL of anhydrous DMF at room temperature. After the mixture was stirred for 15 min, 11 (2 g, 5.7 mmol) was added in small portions. After the mixture was stirred for 1 h, ethyl acetate and water were added, and the organic layer was washed several times with water and then dried over Na₂SO₄. The residue was titrated with hexane/ethyl acetate (10:1, v/v) and filtered to yield 2.47 g (89%) of 12.

6. 6-Methoxy-3-[4-2-(1-piperidinyl)ethoxy]benzo[b]thiophene hydrochloride (13). LiAlH₄ (0.27 g, 7.2 mmol) was added in small portions to a solution of 12 (2.37 g, 4.8 mmol) in 180 mL of anhydrous THF under N₂ at 0 °C. After the mixture was stirred for 30 min, the reaction was quenched by the slow addition of 4 mL of 2.0 M NaOH. The mixture was stirred vigorously for 30 min, and a minimal amount of 2.0 M NaOH was added to dissolve salts. The mixture was then partitioned between water and ethanol/ethyl acetate (1:9, v/v). The aqueous layer was isolated and then extracted several times with ethanol/ethyl acetate (1:9, v/v) and treated with excess diethyl ether to obtain 1.1 g (75%) of 6-hydroxy-3-thiophene hydrochloride (13).
Figure 2. UV spectrum of DMA diquinone methide in 50 mM phosphate buffer (pH 7.4, 37°C). Scans were recorded every 2 s; top scan is the first scan in a time-dependent descending order.

Preparation of DMA Diquinone Methide. Activated silver oxide (29) was prepared by adding KOH (0.72 g in 20 mL of water) to AgNO₃ solution (2.0 g in 20 mL of water), followed by stirring for 15 min. The precipitate was washed with water followed by acetone and then filtered and dried. A mixture of DMA (2 mg, 4.3 μmol), fresh silver oxide (200 mg), and acetonitrile (2 mL) was stirred for 5 min at 60 °C. After filtration, this solution was analyzed immediately using UV or mass spectrometry. DMA diquinone methide: UV (CH₃CN) 216, 6.83 (dd, J = 8.7 Hz, 1H), 7.27 (t, J = 8.9 Hz, 2H), 7.32 (d, J = 2.0 Hz, 1H), 7.73 (q, J = 8.9 Hz, 2H); positive ion electrospray HRMS m/z 464.1706 [M + H]+, calculated for C₂₇H₂₇NO₃FS 464.1696.

Kinetic Studies of the DMA Diquinone Methide. The disappearance of DMA diquinone methide (0.2 mM) in 50 mM K₂HPO₄ buffer (1 mL, pH 7.4, 37 °C) was followed (n = 3) by monitoring the decrease in UV absorbance at 500 nm (2 s/scan) (Figure 2).

Reaction of DMA Diquinone Methide with GSH. A solution of DMA diquinone methide (2 mM) in acetonitrile (1 mL) was combined with GSH (20 mM) in incubation buffer (10 mL, 50 mM phosphate buffer, pH 7.4), and the mixture was stirred at room temperature for 2 min. Perchloric acid (0.2 mL) was added, and the solution was concentrated to remove the remaining acetonitrile under a stream of nitrogen. The final solution was centrifuged for 6 min at 13,000 rpm. The GSH conjugates were purified using semipreparative HPLC (method B).

Oxidation of DMA and 4-F-DMA by Rat or Human Liver Microsomes. Female Sprague–Dawley rats (200–220 g) were obtained from Sasco (Omaha, NE). The rats were pretreated with intraperitoneal injections of dexamethasone, 100 mg/kg daily, for three consecutive days to induce P450 3A isoforms prior to sacrifice on day 4. Rat liver microsomes were prepared, and protein and P450 concentrations were determined as described previously (30). A solution containing the substrate (50 μM), rat liver microsomes (0.34 nmol P450/mg), GSH (0.5 mM), and 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 (31). For control incubations, NADP⁺ or GSH 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 13,000 rpm 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. Human liver microsomes from 10 female donors were obtained from In Vitro Technologies (Baltimore, MD). Incubations with human liver microsomes were carried out using the same procedures as described for the rat liver microsomal incubations.

DNA Damage in Breast Cancer Cell Lines Using the Alkaline Single Cell Gel Electrophoresis Assay (Comet Assay). A viability assay was conducted to determine the dose of the test compounds that gave at least 80% viability during the comet assay. The comet assay was carried out as recommended by the manufacturer ( Trevigen Inc., Gaithersburg, MD). Briefly, the cells (2 × 10⁵ cells/mL) were incubated with the test compounds for 90 min; the attached cells were trypsinized and combined with the suspended cells in the medium by centrifugation. The cells were washed with PBS and resuspended in PBS at a concentration of 2 × 10⁶ cells/mL. Cells (30 μL) were combined with LMAgarose (300 μL, 42 °C), and 50 μL of the mixture was immediately placed onto a CometSlide. The slides were incubated at 4 °C in the dark for 10 min followed by immersion in prechilled lysis solution (pH 10) at 4 °C for 30 min and then incubated in freshly prepared alkali electrophoresis solution (pH > 13) at room temperature in the dark for 45 min. Following electrophoresis in alkali solution at 1 V/cm for 30 min, the slides were immersed in ethanol for 5 min and air-dried. The slides were then stained with SYBR green and viewed under a fluorescence microscope (Nikon Eclipse E600, Nikon Corp., Japan). The DNA was scored from 0 (intact DNA) to 4 (completely damaged DNA with tail only). Scores were calculated using the following formula in which N₀ (intact DNA), N₅, N₆, Nₐ, and Nₑ (completely damaged DNA) were the number of different kinds of comets: score (S) = (N₀ × 0 + N₅ × 1 + N₆ × 2 + Nₐ × 3 + Nₑ × 4)/N₀ + N₅ + N₆ + Nₐ + Nₑ × 100. Duplicated samples were prepared for each treatment, and at least 100 cells were scored per sample.

ER Competitive Binding Assay. The procedure of Obourn et al. (33) was used with minor modifications (34) for the determination of binding of SERMs to ER. Briefly, 24 h prior to the assay, 50% v/v hydroxyapatite slurry was prepared using 10 g of hydroxyapatite in 60 mL of TE buffer (50 mM Tris-Cl, 1 mM EDTA, pH 7.4) and stored at 4 °C. The ER binding buffer consisted of 10 mM Tris-Cl (pH 7.5), 10% glycerol, 2 mM dithiothreitol, and 1 mM Mg/L bovine serum albumin. The ERβ wash buffer contained 40 mM Tris-Cl (pH 7.5) and 100 mM KCl, and the ERα wash buffer was 40 mM Tris-Cl (pH 7.5). A "hot
mix” of 400 nM [3H]estradiol was freshly prepared consisting of 8 nL of 10 μM [3H]estradiol (95 Ci/mmol), 96 nL of ethanol, and 96 μL of ER binding buffer. The reaction mixture consisted of 5 μL of test sample, 5 μL of human recombinant ERβ or ERα (0.5 pmol) (Panvera Corporation, Madison, WI), 5 μL of hot mix, and 85 μL of ER binding buffer. The incubations were carried out at room temperature for 2 h, 100 μL of 50% hydroxylapatite slurry was added, and the tubes were incubated on ice for 15 min with vortexing every 5 min. The appropriate ER wash buffer was added (1 mL), and the tubes were vortexed and centrifuged at 2000 g for 5 min. The supernatant was discarded, and the wash step was repeated two times. The hydroxylapatite pellet containing the ligand—receptor complex was resuspended in 200 μL of ethanol and transferred to scintillation vials. Cytoscent (4 mL) (ICN Radiochemicals, Costa Mesa, CA) was added, and radioactivity was determined by scintillation counting using a Beckman (Schaumburg, IL) LS 5801 liquid scintillation counter. The percent inhibition of [3H]estradiol (% of the samples was calculated in comparison to estradiol (50 nM, 100%). The data represent the average SD of three determinations.

Inhibition of Estradiol-Induced Alkaline Phosphatase Activity in Ishikawa Cells. The procedure of Pisha and Pezzuto was used as described previously (35). Briefly, Ishikawa cells (5 × 10⁴ cells/mL) were incubated overnight with estrogen-free media in 96 well plates. Test samples (with 0.5 nM 17β-estradiol) and appropriate controls were added. The 0 day control did not contain any additional estradiol. The cells were incubated with a total volume of 200 μL/well at 37 °C for 4 days. The cells were washed three times with PBS and lysed by freeze—thawing in the presence of 0.1 M Tris, pH 8.0. Enzyme activity was measured by reading the liberation of p-nitrophenol from 100 μM p-nitrophenolphosphate at 340 nm every 15 s for 16–20 readings with an ELISA reader (Power Wave 200 microplate scanning spectrophotometer, Bio-Tek Instrument, Winooski, VT). The maximum slope of the lines generated by the kinetic readings was calculated using a Kinecalc computer program (Bio-Tek Instrument). For antiestrogenic activity, the reduction in percent induction as compared to the DMSO control was determined as follows: [(slopeDMSO − slopeDMSO blank)/(slopeDMSO − slopeDMSO blank)] × 100. The data represent the average SD of three determinations.

Results

Synthesis of DMA and 4′-F-DMA. DMA was synthesized according to the method of Palkowitz et al. (28) with modifications (Scheme 1). Bromination of 6-methoxy-2-(4-methoxyphenyl)-3-benzo[4]thiophene, 1, was attempted with Br2/CHCl3 at 60 °C; however, a mixture of brominated products at different positions resulted. The desired product 2 could not be purified by column chromatography or recrystallization; therefore, an alternative method for the bromination of 1 was developed with N-bromoacetic acid as the bromination reagent, which provided the 3-bromo derivative exclusively and in quantitative yield. During the synthesis of 4′-F-DMA in the cyclization—rearrangement step catalyzed by polyphosphoric acid, different reaction temperatures caused different major products. If the reaction was carried out at 120 °C, the major product was the desired rearranged product, 6-methoxy-2-(4-fluorophenyl)benzo[b]thiophene; however, if the reaction was carried out at 100 °C, the major product became the unrearranged product, 6-methoxy-3-(4-fluorophenyl)benzo[b]thiophene.

Kinetic Studies of DMA Diquinone Methide. Mild oxidants such as silver(I) oxide (29), lead(IV) oxide (36, 37), and manganese(IV) oxide (38) have been used for the efficient synthesis of quinones and quinone methides from various phenolic compounds. In this study, we used silver oxide as the chemical oxidant to generate the DMA diquinone methide from DMA. Absent from the spectrum of DMA itself, a strong absorbance at 490 nm was observed in the UV spectrum of the DMA diquinone methide in acetonitrile, which is similar to the UV spectrum of the raloxifene diquinone methide (18). Furthermore, a protonated molecular of m/z 460 was observed in the positive ion electrospray mass spectrum of DMA diquinone methide, which was two mass units less than that of DMA. Based on the measurement of the rate of disappearance of the DMA diquinone methide (Figure 2), the half-life of the DMA diquinone methide at physiological pH and temperature was determined to be 15.0 ± 0.4 s.

Reaction of DMA Diquinone Methide with GSH. The DMA diquinone methide, formed using silver oxide, reacted with GSH to give seven GSH conjugates: one tri-GSH conjugate, two di-GSH conjugates, and four mono-GSH conjugates. Four mono-GSH conjugates (Figure 3) were arbitrarily assigned as DMA-SG1, DMA-SG2, DMA-SG3, and DMA-SG4. During MS-MS with CID of the protonated molecules of m/z 767, DMA-SG1, DMA-SG2, and DMA-SG3 showed similar fragmentation patterns. For example, all produced a fragment ion of m/z 638, corresponding to loss of a γ-glutamyl group (Figure 4). The product ion of m/z 749 was formed by elimination of water from the protonated molecule, and the fragment ion of m/z 112 (protonated vinylpiperidinone) was indicative of the presence of an intact ethylpiperidine moiety in the conjugates. The product ion of m/z 494 was probably formed by a cleavage adjacent to the thioether moiety with charge retention on the DMA residue. This type of fragmentation and the loss of a γ-glutamyl group are characteristic of glutathione conjugates (39).

For DMA-SG4, MS-MS with CID of the protonated molecules of m/z 548 produced ions of m/z 473, 419, and 130, corresponding to fragmentation of glutathione moiety (Figure 5). The product ion of m/z 273 was probably formed by a cleavage adjacent to the thioether moiety with charge retention on the DMA residue. The di-GSH conjugates, DMA-diSG1 (Figure 3) produced a protonated molecule of m/z 853. MS-MS with CID of this protonated molecule produced fragment ions of m/z 724 and 595, corresponding to losses of one and two γ-glutamyl groups from each GSH (data not shown). DMA-diSG2 (Figure 3) produced an ion of m/z 536.5 corresponding to [M + 2H]⁺, which gave a similar fragmentation pattern corresponding to loss of the glutamyl groups (m/z 472, 407.5). DMA-triSG1 produced a triply charged ion [M + 3H]⁻ of m/z 460.

Four mono-GSH conjugates were isolated using semi-preparative HPLC and characterized using proton NMR, and DMA was analyzed for comparison (Table 1). In the spectrum of DMA, the doublet signals at 7.53 and 6.73 ppm were assigned to the two pairs of equivalent protons on the phenol moiety (a and b in Figure 1), while the quartet signal at 6.89 ppm was assigned to the two pairs of protons on the O-alkylated phenol ring (c and d in Figure 1). A doublet at 6.76 ppm was attributed to proton 5 on the benzothiophene moiety, which correlated with proton 4 and proton 7 in COSY spectrum. Consequently, both protons 4 and 7 were represented by doublet signals with different coupling constants. In the case of DMA-SG1, all signals associated with the benzo-
[b]thiophene (proton 4, 5, and 7) and O-alkylated phenol moieties (c and d) remained intact; however, signals corresponding to protons a and b were replaced by those representing e, f, and g. These features led to the assignment of this metabolite as 3′-glutathionyl-DMA. For DMA-SG2, two singlets at 6.98 and 7.02 ppm were consistent with a para relationship between protons 4 and 7; no correlation between proton 4 and proton 7 was observed in COSY spectrum, and therefore, this metabolite was assigned as 5-glutathionyl-DMA. For DMA-SG3, there are two doublets at 6.94 and 7.24 ppm, which indicated an ortho relationship between protons 4 and 5; a correlation between proton 4 and 5 was also observed in the COSY spectrum, and thus this metabolite was identified as 7-glutathionyl-DMA. For DMA-SG4, all signals associated with the benzothiophene (protons 4, 5, and 7) and phenol moieties (a and b) remained intact; however, the quartet signal at 6.90 ppm was missing indicating that GSH replaced the O-alkylated phenol moiety to form a 3-glutathionyl-DMA. The di-GSH conjugates and tri-GSH conjugate were too unstable for isolation and NMR characterization.
Incubation of DMA and 4-F-DMA with Liver Microsomes and GSH. Four mono-GSH conjugates and one di-GSH conjugate obtained from the microsomal incubations of DMA with GSH (Figure 6) were identical to the GSH conjugates generated by the reaction of DMA diquinone methide with GSH. In addition to the DMA diquinone methide GSH conjugates, two DMA catechols, two DMA o-quinone mono-GSH conjugates, and two DMA o-quinone di-GSH conjugates were detected by LC-MS-MS; however, the yields were so low that the DMA o-quinone GSH conjugates could not be detected by UV.

The two DMA catechols gave protonated molecules at \( m/z \) 478, and the two DMA o-quinone mono-GSH conjugates gave protonated molecules at \( m/z \) 783. Subsequent CID of the protonated molecules at \( m/z \) 783 produced fragment ions at \( m/z \) 654, corresponding to loss of one pyroglutamate, which is characteristic for GSH-conjugated metabolites (40). The two DMA o-quinone di-GSH conjugates gave a doubly charged ion at \( m/z \) 544.5 [M + 2H]\(^{2+}\). Subsequent CID of the doubly charged ion at \( m/z \) 544.5 produced fragment ions at \( m/z \) 480, corresponding to loss of one pyroglutamate. In the human liver microsomal incubations of DMA in the presence of GSH, almost the same GSH conjugates generated by rat liver microsomal incubations were obtained. Incubation with 4-F-DMA and either rat or human liver microsomes gave no detectable formation of GSH conjugates (Figure 6).

Reactions of DMA Diquinone Methide with Deoxynucleosides. DMA diquinone methide generated by chemical oxidation was incubated with four deoxynucleosides at physiological pH and temperature. One DMA diquinone methide deoxyguanosine adduct was detected by LC/MS. MS-MS with CID showed the product ion of [MH – 116]\(^{+}\) resulting from loss of the sugar (data not available).

### Table 1: Proton NMR Data for DMA and Its GSH Conjugates

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<th>H4</th>
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<th>H7</th>
<th>Ha</th>
<th>Hb</th>
<th>He</th>
<th>Hd</th>
<th>He</th>
<th>Hf</th>
<th>Hg</th>
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<tr>
<td>DMA(^a)</td>
<td>7.13, d</td>
<td>6.76, dd</td>
<td>7.18, d</td>
<td>7.53, d</td>
<td>6.73, d</td>
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<td>7.05, dd</td>
<td>7.28, d</td>
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<td>7.07, d</td>
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<td>7.02, s</td>
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<td>DMA(^b)</td>
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<td>6.77–6.81, m</td>
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<td>DMA-SG4(^b)</td>
<td>7.73, d</td>
<td>6.95, bd</td>
<td>7.26, bs</td>
<td>7.51, d</td>
<td>6.86, d</td>
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\(^a\) CD\(_3\)OD. \(^b\) DMSO-\(d_6\).
In the present study, the results showed (Figure 7) that DMA diquinone methide was successfully generated by chemical oxidation using silver oxide and characterized by UV and mass spectrometry. Kinetic studies showed that the half-life of the DMA diquinone methide was 15.0 ± 0.4 s at physiological pH and temperature, which is considerably more stable than the raloxifene diquinone methide (t1/2 < 1 s) (18). The only structural difference between DMA and raloxifene is the different substitution at the 3 position; the phenoxyl group of DMA is an electron-donating resonance group, which stabilized the DMA diquinone methide, whereas raloxifene possesses an electron-withdrawing carbonyl group, which largely destabilized the raloxifene diquinone methide.

Three GSH conjugates of raloxifene, identified by Chen et al. (17), were the 3′-, 5′-, and 7-glutathionyl raloxifene adducts, after incubation with human liver microsomes. It was suggested that either a raloxifene epoxide or raloxifene diquinone methide may be involved in the formation of these GSH adducts. DMA is predicted to form similar GSH conjugates in the presence of rat and human liver microsomes. Four mono-GSH conjugates and one di-GSH conjugate obtained in microsomal incubations were identical to the GSH conjugates observed in the reaction of DMA diquinone methide with GSH, which supports the fact that the DMA diquinone methide is the reactive intermediate in the formation of DMA–GSH conjugates in microsomal incubations. Based on LC-MS-MS and NMR analyses, after incubation with rat liver microsomes, three mono-GSH conjugates of DMA (DMA-SG1, DMA-SG2, and DMA-SG3) were identified: 3′-, 5′-, and 7-glutathionyl DMA (Scheme 3). DMA-SG4 was identified as 3-glutathionyl DMA; no corresponding conjugate is seen in the microsomal incubation of raloxifene. This conjugate results from Michael-type addition to the diquinone methide intermediate at the 3-position and displacement of a phenolate leaving group, a pathway not possible for raloxifene (Scheme 4). The detection of 4-[2-(piperidinyl)ethoxy] phenol by LC-MS-MS from DMA incubations with rat liver microsomes further confirmed the release of the 4-[2-(piperidinyl)ethoxy]-phenoxyl leaving group in the formation of DMA-SG4 (data not shown).

DMA o-quinone GSH conjugates were also detected using LC-MS-MS in the incubation of DMA with rat liver microsomes, although the yields were very low. In the human liver microsomal incubation of DMA in the presence of GSH, the same GSH conjugates generated by rat liver microsomes were observed.

Only one DMA diquinone methide deoxyguanosine adduct was detected by LC-MS-MS in the reaction of DMA diquinone methide with any of the four deoxy-nucleosides at physiological pH and temperature. Since the lifetime of the DMA diquinone methide is short (t1/2 = 15 s), it is likely that most of the DMA diquinone

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**Discussion**

In this study, DMA diquinone methide was successfully generated by chemical oxidation using silver oxide and characterized by UV and mass spectrometry. Kinetic studies showed that the half-life of the DMA diquinone methide was 15.0 ± 0.4 s at physiological pH and temperature, which is considerably more stable than the raloxifene diquinone methide (t1/2 < 1 s) (18). The only structural difference between DMA and raloxifene is the different substitution at the 3 position; the phenoxyl group of DMA is an electron-donating resonance group, which stabilized the DMA diquinone methide, whereas raloxifene possesses an electron-withdrawing carbonyl group, which largely destabilized the raloxifene diquinone methide.

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methide decayed before it reacted with deoxynucleosides, which are much less nucleophilic compared to GSH. DMA induced dose-dependent DNA damage and caused more DNA damage than raloxifene in S30 (ER$^+$) cell lines. These results suggest that DMA could induce ER-independent DNA damage in vivo.

Based on previous structure–activity studies of raloxifene (42), small, highly electronegative 4′-substituents such as hydroxyl and fluoro are preferred in both in vitro and in vivo assays. 4′-F-DMA was designed to retain the ER activity of DMA but to be devoid of the propensity to form the reactive diquinone methide metabolite. In both rat and human liver microsomal incubations of 4′-F-DMA in the presence of GSH, no GSH conjugates were detected (Figure 6), which indicated that the replacement of the 4′-hydroxyl group with fluoro successfully blocked the formation of the DMA diquinone methide. In the comet assay, 4′-F-DMA induced much less DNA damage than DMA. The ER binding affinity and the antiestrogenic activity in Ishikawa cells of 4′-F-DMA were also investigated. The results showed that 4′-F-DMA and DMA have similar ER binding affinity and that 4′-F-DMA has a 10-fold lower antiestrogenic activity than DMA, but a comparable antiestrogenic activity to raloxifene (Table 2).

In conclusion, DMA can be oxidized chemically or enzymatically to a diquinone methide that reacts with GSH or deoxyguanosine to form adducts at physiological pH and temperature. In the rat and human liver microsomal incubations, small amounts of DMA o-quinone GSH conjugates were also detected. DMA induced dose-dependent DNA damage in the ER$^+$ S30 cell line. These data suggest that DMA quinoids could contribute to cytotoxicity and genotoxicity through depletion of GSH and induction of DNA damage. 4′-F-DMA was designed to reduce toxicity while retaining biological activity. In
micronosomal incubations of 4′-F-DMA, no GSH adducts were detected, and 4′-F-DMA induced less DNA damage as compared to DMA using comet assay, whereas 4′-F-DMA showed similar ER binding affinity to DMA itself, and a similar antiestrogenic activity to raloxifene. Therefore, 4′-F-DMA demonstrates that SERMs may be designed with attenuated toxicity that maintain ER binding and ER modulating activity.

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References


