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In vitro bioactivation of bazedoxifene and 2-(4-hydroxyphenyl)-3-methyl-1*H*-indol-5-ol in human liver microsomes

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ABSTRACT

Bazedoxifene is a selective estrogen receptor modulator (SERM) that has been developed for use in post-menopausal osteoporosis. However, it contains a potentially toxic 5-hydroxy-3-methylindole moiety. Previous studies on the 5-hydroxyindole and the 3-alkylindole-containing drugs indometacine, zafirlukast and MK-0524 structural analogs have shown that they are bioactivated by cytochrome P450s through a dehydrogenation process to form quinoneimine or 3-methyleneindolenine electrophilic species. In the present study, bazedoxifene was synthesized and then evaluated, together with raloxifene and 2-(4-hydroxyphenyl)-3-methyl-1*H*-indol-5-ol (**13**), a 3-methyl-5-hydroxyindole-based structural fragment of bazedoxifene, for its ability to form reactive electrophilic species when incubated with human liver microsomes (HLMs) or recombinant CYP isozymes. We showed that bazedoxifene was bioactivated only in trace amounts with recombinant CYP isozymes. In contrast, the *N*-dealkylated fragment of bazedoxifene (2-(4-hydroxyphenyl)-3-methyl-1*H*-indol-5-ol) was bioactivated in considerable amounts to an electrophilic intermediate, which was trapped with glutathione and identified by LC–MS/MS. This suggests that bazedoxifene would require initial *N*-dealkylation, which could subsequently lead to the formation of the reactive intermediate. However, such an *N*-dealkylated metabolite of bazedoxifene was not detected after the incubation of bazedoxifene in HLM or recombinant CYP isozymes.

1. Introduction

Raloxifene **I** (Fig. 1), a benzothiophene selective estrogen receptor modulator (SERM), is currently the only FDA-approved SERM available for the prevention and treatment of osteoporosis in post-menopausal women [1]. It acts as an estrogen receptor agonist in bone tissue, while not stimulating the breast and uterine tissues [2,3]. Its administration is associated with a number of side-effects of which hot flushes, leg cramps, and thromboembolism are the most severe [5,6]. The binding of a SERM to an estrogen receptor triggers the same sequence of molecular events as observed after the binding of estrogen itself [4]. The agonist or antagonist activity is tissue-dependent, so the distribution of estrogen receptors in various tissues becomes important since modulators of estrogen function can act not only on bone tissue but also on all other tissues where estrogen exerts its effects. It is, therefore, desirable that SERMs should act as agonists preferentially on bone tissue and in order to circumvent negative, undesired effects in the prevention and treatment of post-menopausal osteoporosis.

Bazedoxifene (**II**, Fig. 1) is a new, third generation, indole-based estrogen receptor (ER)-ligand developed for use in post-menopausal osteoporosis. Preclinical studies have revealed that bazedoxifene induces significantly lower uterine stimulation (lower promotion of endometrium cell proliferation) than raloxifene, making it a promising alternative for treating post-menopausal osteoporosis with an improved safety profile [5,6]. Glucuronidation is the major pathway for the metabolism of bazedoxifene, with bazedoxifene-5-glucuronide being the main metabolite [7]. However, no data are available on the formation of the reactive metabolites of bazedoxifene, so we have investigated the potential formation of such reactive intermediates through glutathione (GSH) trapping studies.

The production of reactive metabolites following biotransformation by cytochrome P450 (CYP450) enzymes can lead to toxicity, since they can bind covalently to proteins and/or DNA with toxic consequences. Not only this, but when such metabolites bind covalently to CYP450 they can cause its mechanism-based inactivation (MBI) [8]. Raloxifene, for example, is a mechanism-based inhibitor of cytochrome P450 3A4 (CYP3A4) [9]. The diquinone methide formed after the bioactivation of raloxifene (Fig. S1) alkylates the Cys239 residue of CYP3A4 leading to an irreversible loss of enzyme function [10]. Raloxifene is known to undergo a CYP3A4-catalyzed reaction with its phenolic groups in HLM in the presence of NADPH, such that it yields a reactive diquinone methide and an *o*-quinone species [11]. However, in vivo, glucuronidation of the same phenolic groups in the gut and liver constitutes the principal elimination mechanism of raloxifene in humans [12].

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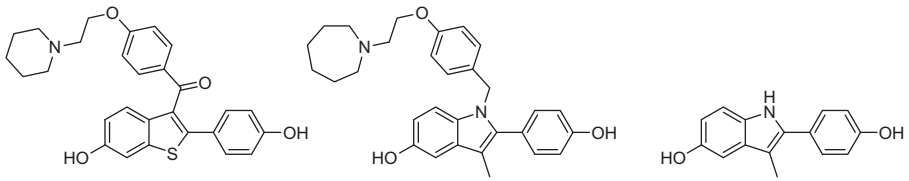


Fig. 1. Structures of raloxifene, bazedoxifene and the *N*-dealkylated fragment of bazedoxifene (2-(4-hydroxyphenly)-3-methyl-1*H*-indol-5-ol).

GSH trapping reactions can provide an insight into the mechanisms of drug bioactivation and the subsequent formation of reactive metabolites in the body. It is common practice for drugs under investigation for the formation of reactive metabolites to be incubated with HLM and GSH. The nucleophilic cysteine sulfhydryl group of GSH can trap any electrophilic intermediate species in the form of S-linked conjugates with GSH that can be analyzed by LC–MS/MS [13]. However, interpretation of the output from these assays is difficult as many successfully marketed drugs also show positive results in this assay [14,15]. Although the GSH trapping of electrophilic drug metabolites formed by HLM is a common method by which potential reactive and toxic metabolites can be identified, it can yield misleading and false positive results. For instance, raloxifene has been found to form potent electrophilic intermediates in vitro [9,12], but such intermediates have never been observed in vivo, probably because raloxifene is conjugated with glucuronic acid extremely quickly in vivo [16]. Bazedoxifene glucuronidation, on the other hand, is a significantly slower process [7,16], meaning the possibility of its electrophilic metabolites' production by the CYP system is increased and warrants our investigation.

Bazedoxifene possesses a 5-hydroxy-3-methyl-substituted indole moiety (Fig. S2). Previous studies on the bioactivation of 5-hydroxyindole to reactive quinoneimine intermediates (Fig. S2) have been reported for oxypertine and indomethacin, both of which have been implicated in numerous cases of aplastic anemia, agranulocytosis and death [17]. 5-Hydroxyindoles are bioactivated by myeloperoxidase or by the CYP450 system to the electrophilic quinoneimine, which can be trapped by GSH. No formalized structure–bioactivation relationship studies have been reported on 5-hydroxyindoles that address their bioactivation.

Previous studies on 3-methylindole, which is produced in animal and human digestive systems through tryptophan degradation and is found in cigarette smoke [18], have demonstrated that it is a potent pneumotoxin that can cause severe lung injury. 3-Methylindole is bioactivated by CYP450s to form a reactive electrophilic intermediate through the dehydrogenation pathway (Fig. S2) [19–22]. Their toxicity has not been fully evaluated in humans, but the compounds are a substrate for several human CYP450 enzymes. The toxicity of the 3-methylindole attributed to the action of 3-methyleneindolenine obtained by dehydrogenation of the 3-methylindole (Fig. S2) and its adducts with thiol nucleophiles upon incubation with microsomes have been observed. 3-Alkylindole-containing drugs (see Fig. S3 for structures), such as the leukotriene receptor antagonist zafirlukast [13,23], structural analogs of MK-0524 [24,25], and the TNF-α antagonist SPD-304 [26], are also bioactivated by P450s through a similar dehydrogenation pathway to electrophilic 3-methyleneindolenine intermediates that are trapped with GSH. 3-Methylindole-like bioactivation has also been reported on a 3-substituted-7-azaindole derivative L-745,870, intended for the treatment of schizophrenia [27]. It has been proposed that the formation of the electrophilic 3-methyleneindolenine is a two-step process in which dehydrogenation was initiated either by hydrogen atom abstraction from the 3-methyl or 3-methylene carbon, or by electron abstraction from the indole nitrogen atom, followed by a second one-electron oxidation. The dehydrogenation atom abstraction step was believed to be the rate-limiting step of the 3-methylindole dehydrogenation, but either a hydrogen atom abstraction or a nitrogen oxidation was the potential initiating step for the dehydrogenation of zafirlukast and analogs of MK-0524 [23–25].

The aim of the present study was to determine whether bazedoxifene can be dehydrogenated to an electrophilic intermediate by CYP450 enzyme(s) in HLM. We describe the synthesis of bazedoxifene and an *N*-dealkylated fragment of bazedoxifene **13** (Scheme 1), and the GSH trapping studies on bazedoxifene, the structurally related raloxifene, and 2-(4-hydroxyphenyl)-3-methyl-1*H*-indol-5-ol (**13**), a 5-hydroxy-3-methylindole-based structural fragment of bazedoxifene, in the presence of HLM or different recombinant CYP450 isozymes.

2. Materials and methods

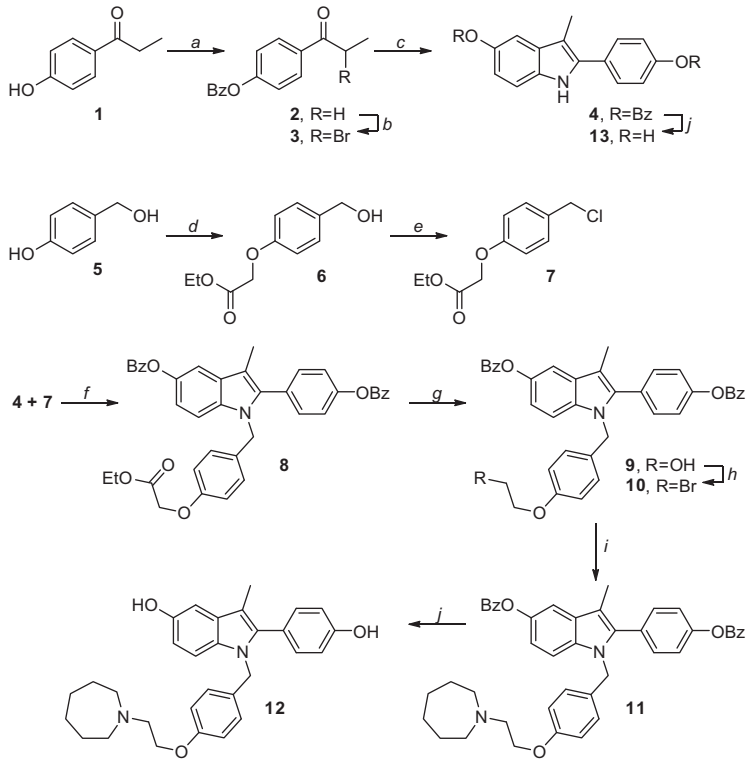
2.1. Synthesis of bazedoxifene

Chemicals were obtained from Acros, Aldrich Chemical Co. and Fluka and used without further purification. Yields refer to purified products and were not optimized. Analytical thin-layer chromatography (TLC) was performed using silica gel $60F_{254}$ pre-coated plates (0.25 mm thick) with a fluorescent indicator from Merck (Germany). Flash column chromatography was carried out on silica gel 60 (particle size 0.040–0.063 mm; Merck, Germany). The components of chromatographic eluents are given as volume-to-volume ratios (v/v). ^{1}H NMR spectra were recorded at 300 MHz on a Bruker AVANCE DPX_{300} spectrometer in $CDCl_3$ or DMSO-d_6 solution with TMS as the internal standard. Mass spectra were obtained using a VG-Analytical Autospec Q mass spectrometer.

The purity of the synthesized bazedoxifene (**12**) was determined by chromatographic separation on an Agilent 1100 system (Agilent Technologies, Santa Clara, USA) using a Kinetex 50 mm × 2.1 mm column coupled with an InLine filter KrudKatcher Ultra HPLC 0.5 mm and a guard column C18 4 mm × 2 mm (Phenomenex, Torrance, USA) at room temperature and with UV detection at 295 nm. The mobile phase consisted of 0.1% formic acid in water (mobile phase A) and 100% acetonitrile (mobile phase B). The flow rate was set at 0.5 mL/min and the separation required gradient elution. Elution started with 25% of mobile phase B, and continued with the following gradient: 25–63–25–25% of mobile phase B in 0.06–6.00–6.20–12.00 min. The run time was 12 min and the volume of injection was 5 μL.

2.1.1. Synthesis of 1-(4-(benzyloxy)phenyl)propan-1-one (*2*)

Benzyl bromide (16.0 mL, 0.135 mol) was slowly added to a stirred solution of 1-(4-hydroxyphenyl)propan-1-one (**1**) (20.00 g, 0.133 mol) and potassium carbonate (18.36 g, 0.133 mol) in acetone (200 mL). Having been stirred at 60 °C overnight, the reaction mixture was cooled, the precipitate then being filtered off and the solvent evaporated under reduced pressure. The residue was dis-



Scheme 1. Reagents and conditions: (a) benzyl bromide, K_2CO_3, acetone, 60 °C, overnight; (b) Br_2, glacial acetic acid, r.t., 3 h; (c) (i) 4-benzyloxyaniline hydrochloride, Et_3N, DMF, 120 °C, 2 h; (ii) 4-benzyloxyaniline hydrochloride, 150 °C, 2 h; (d) ethyl bromoacetate, K_2CO_3, acetonitrile, 60 °C, overnight; (e) $SOCl_2$, DMF, dichloromethane, 0 °C, 45 min; (f) NaH, DMF, 0 °C to r.t., overnight; (g) $LiAlH_4$, THF, 0 °C, 30 min; (h) CBr_4, PPh_3, THF, r.t., overnight; (i) hexamethylenimine, THF, reflux, overnight; and (j) H_2, Pd/C, EtOH/THF, r.t., overnight.

solved in ethyl acetate (300 mL), then washed with water (2 × 200 mL) and brine (200 mL). The organic phase was dried over Na_2SO_4, filtered and the solvent removed *in vacuo*. Yield: 100.0%; white solid; mp 98–100 °C ([28], 102–103 °C); ^{1}H NMR ($CDCl_3$): δ = 1.24 (t, 3H, J = 7.3 Hz, $CH_2C\underline{H}_3$), 2.97 (q, 2H, J = 7.3 Hz, $C\underline{H}_2CH_3$), 5.15 (s, 2H, $PhC\underline{H}_2$), 7.03 (d, 2H, J = 8.9 Hz, 2 × Ar-H), 7.31–7.48 (m, 5H, Ph), 7.97 (d, 2H, J = 8.9 Hz, 2 × Ar-H) ppm.

2.1.2. Synthesis of 1-(4-(benzyloxy)phenyl)-2-bromopropan-1-one (**3**)

Compound **2** (22.5 g, 0.094 mol) was suspended in glacial acetic acid (70 mL) and cooled to 0 °C. A solution of bromine (4.8 mL, 0.094 mol) in glacial acetic acid (20 mL) was then added dropwise and the reaction mixture stirred for 3 h at room temperature. Water (200 mL) was then added to the reaction mixture and the product extracted with diethyl ether (2 × 200 mL). The combined organic extracts were washed successively with water (200 mL), saturated aqueous $NaHCO_3$ solution (200 mL) and brine (200 mL). The organic phase was dried over Na_2SO_4, filtered and the solvent evaporated under reduced pressure. The crude product was recrystallized from a mixture of hexane and ethyl acetate. Yield: 32.2%; white solid; mp 71–75 °C ([28], 75–76 °C); ^{1}H NMR ($CDCl_3$): δ = 1.91 (d, 3H, J = 6.6 Hz, $CHC\underline{H}_3$), 5.17 (s, 2H, $PhC\underline{H}_2$), 5.27 (q, 1H, J = 6.6 Hz, $C\underline{H}CH_3$), 7.05 (d, 2H, J = 9.0 Hz, 2 × Ar-H), 7.31–7.50 (m, 5H, Ph), 8.03 (d, 2H, J = 9.0 Hz, 2 × Ar-H) ppm.

2.1.3. Synthesis of 5-(benzyloxy)-2-(4-(benzyloxy)phenyl)-3-methyl-1H-indole (**4**) [29]

4-Benzyloxyaniline hydrochloride (4.001 g, 16.0 mmol) was added to a solution of compound **3** (3.964 g, 12.4 mmol) in *N,N*-dimethylformamide (15 mL), following which the reaction mixture was purged with argon for 10 min. Triethylamine (4.3 mL, 30.9 mmol) was then added and the reaction mixture heated at 120 °C for 2 h. The reaction mixture was cooled and additional 4-benzyloxyaniline hydrochloride (4.100 g, 16.4 mmol) was added before the reaction mixture was heated to 150 °C for 2 h. After the reaction's completion, the mixture was cooled to room temperature and poured into water (200 mL) before the product was extracted with ethyl acetate (3 × 70 mL). The combined organic extracts were washed successively with 1 M NaOH (100 mL), water (100 mL) and brine (100 mL), prior to being dried over Na_2SO_4, filtered and the solvent being evaporated under reduced pressure. The crude solid product was first washed with methanol (50 mL) and filtered then washed with diethyl ether (50 mL) and filtered. Yield: 84.9%; white solid; mp 146–150 °C ([29], 150–152 °C); ^{1}H NMR ($CDCl_3$): δ = 2.41 (s, 3H, CH_3), 5.15 (d, 2H, $PhC\underline{H}_2$), 5.17 (d, 2H, $PhC\underline{H}_2$), 6.95 (dd, 1H, ^3J = 2.4, ^2J = 8.7 Hz, Ar-H-6), 7.10 (d, 2H, J = 8.8 Hz, 2 × Ar-H′), 7.13 (d, 2H, ^3J = 2.4 Hz, Ar-H-4), 7.26 (d, 1H, ^2J = 8.7 Hz, Ar-H-7), 7.29–7.53 (m, 12H, 2 × Ph, 2 × Ar-H′), 7.84 (s, 1H, NH) ppm.

2.1.4. Synthesis of ethyl 2-(4-(hydroxymethyl)phenoxy)acetate (**6**)

A solution of 4-hydroxybenzyl alcohol (**5**) (15.32 g, 12.0 mmol), potassium carbonate (17.10 g, 48.0 mmol) and ethyl bromoacetate (15.1 mL, 12.6 mmol) in acetonitrile (150 mL) was stirred at 60 °C overnight. Once the solvent was evaporated, the residue was dissolved in ethyl acetate (100 mL) and washed successively with 10% aqueous citric acid solution (2 × 50 mL), saturated aqueous $NaHCO_3$ solution (2 × 50 mL) and brine (50 mL). It was subsequently dried over Na_2SO_4, filtered and the solvent removed *in vacuo*. The crude product was purified with flash column chromatography using dichloromethane/methanol (20:1) as the eluent. Yield: 65%; brown oil; IR (KBr): ν = 2998, 1704, 1615, 1588, 1383, 1212, 1083, 1030, 949, 789, 708 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ = 1.27 (t, 3H, J = 7.1 Hz, $CH_2C\underline{H}_3$), 2.58 (s, 1H, OH), 4.23

(q, 2H, J = 7.1 Hz, C$\underline{H_2}$CH$_3$), 4.54 (s, 2H, CH$_2$), 4.57 (s, 2H, CH$_2$), 6.85 (d, 2H, J = 8.7 Hz, 2 × Ar-H), 7.24 (d, 2H, J = 8.7 Hz, 2 × Ar-H) ppm.

*2.1.5. Synthesis of ethyl 2-(4-(chloromethyl)phenoxy)acetate (**7**)*

A solution of compound **6** (14.08 g, 67 mmol) and *N,N*-dimethylformamide (1 mL) in dichloromethane (200 mL) under an argon atmosphere was cooled to 0 °C and thionyl chloride (5.4 mL, 74 mmol) dissolved in dichloromethane (80 mL) was added dropwise. The reaction mixture was stirred at 0 °C for 45 min and the solvent evaporated under reduced pressure. Yield: 100%; pale yellow oil; IR (KBr): ν = 2977, 1754, 1652, 1613, 1588, 1514, 1440, 1113, 1027, 833, 733, 665 cm^{-1}. ^{1}H NMR (CDCl$_3$): δ 1.31 (t, 3H, J = 7.1 Hz, CH$_2$C$\underline{H_3}$), 4.29 (q, 2H, J = 7.1 Hz, C$\underline{H_2}$CH$_3$), 4.57 (s, 2H, CH$_2$), 4.63 (s, 2H, CH$_2$), 6.90 (d, 2H, J = 8.7 Hz, 2 × Ar-H), 7.33 (d, 2H, J = 8.7 Hz, 2 × Ar-H) ppm.

*2.1.6. Synthesis of 4-(5-(benzoyloxy)-1-(4-(2-ethoxy-2-oxoethoxy)benzyl)-3-methyl-1H-indol-2-yl)phenyl benzoate (**8**) [29]*

A solution of compound **4** (4.213 g, 9.41 mmol) in *N,N*-dimethylformamide (40 mL) was cooled to 0 °C on an ice bath and sodium hydride (0.29 g, 12.1 mmol) was added. The reaction mixture was stirred for 20 min, followed by the addition of compound **7** (3.440 g, 15.0 mmol). After stirring at room temperature overnight, the solvent was evaporated under reduced pressure and the residue dissolved in ethyl acetate (100 mL). The organic phase was washed with water (2 × 100 mL) and brine (100 mL), dried over Na_2SO_4, and filtered. The solvent was then evaporated under reduced pressure. The crude product was purified by flash column chromatography using ethyl acetate/petroleum ether (1:4) as the eluent. Yield: 29.7%; yellow solid; mp 128–131 °C ([29], 129–131 °C); ^{1}H NMR (DMSO-d_6): δ = 1.18 (t, 3H, J = 7.1 Hz, CH$_2$C$\underline{H_3}$), 2.16 (s, 3H, CH$_3$), 4.13 (q, 3H, J = 7.1 Hz, C$\underline{H_2}$CH$_3$), 4.67 (s, 2H, CO-C$\underline{H_2}$O), 5.12 (s, 2H, CH$_2$), 5.15 (s, 2H, CH$_2$), 5.17 (s, 2H, CH$_2$), 6.75 (s, 4H, 4 × Ar-H″), 6.82 (dd, 1H, ^3J = 2.4 Hz, ^2J = 8.7 Hz, Ar-H-6), 7.11–7.14 (m, 3H, 2 × Ar-H′, Ar-H-4), 7.21 (d, 1H, ^2J = 8.7 Hz, Ar-H-7), 7.31 (d, 2H, J = 8.5 Hz, 2 × Ar-H′), 7.34–7.50 (m, 10H, 2 × Ph) ppm.

*2.1.7. Synthesis of 2-(4-((5-(benzyloxy)-2-(4-(benzyloxy)phenyl)-3-methyl-1H-indol-1-yl)methyl)phenoxy)ethanol (**9**) [29]*

A solution of compound **8** (1.700 g, 2.66 mmol) in dry tetrahydrofuran (20 mL) was cooled to 0 °C and 1 M solution of $LiAlH_4$ in tetrahydrofuran (3.1 mL, 3.10 mmol) was added dropwise. After 30 min, the reaction was slowly quenched with water and tetrahydrofuran was evaporated under reduced pressure. The residue was then partitioned between 1 M HCl (20 mL) and ethyl acetate (20 mL). The organic phase was dried over Na_2SO_4, filtered and the solvent evaporated under reduced pressure. The product was used in the next step without further purification. Yield: 73.3%; yellow solid; mp 107–111 °C; ^{1}H NMR (DMSO-d_6): δ = 2.16 (s, 3H, CH$_3$), 3.65 (t, 2H, J = 5.0 Hz, CH$_2$C$\underline{H_2}$OH), 3.88 (t, 2H, J = 5.0 Hz, C$\underline{H_2}$CH$_2$OH), 5.12 (s, 2H, CH$_2$), 5.15 (s, 2H, CH$_2$), 5.16 (s, 2H, CH$_2$), 6.75 (s, 4H, 4 × Ar-H″), 6.82 (dd, 1H, ^3J = 2.4 Hz, ^2J = 8.8 Hz, Ar-H-6), 7.11–7.14 (m, 3H, 2 × Ar-H′, Ar-H-4), 7.21 (d, 1H, ^2J = 8.8 Hz, Ar-H-7), 7.30 (d, 2H, J = 8.7 Hz, 2 × Ar-H′), 7.34–7.50 (m, 10H, 2 × Ph) ppm.

*2.1.8. Synthesis of 4-(5-(benzoyloxy)-1-(4-(2-bromoethoxy)benzyl)-3-methyl-1H-indol-2-yl)phenyl benzoate (**10**) [29]*

CBr_4 (0.970 g, 2.92 mmol) and PPh_3 (0.760 g, 2.92 mmol) were added to a solution of compound **9** (1.107 g, 1.85 mmol) in dry tetrahydrofuran (30 mL). The reaction mixture was stirred at room temperature overnight and the solvent then evaporated under reduced pressure. The crude product was purified by flash column chromatography using ethyl acetate/hexane (1:4) as the eluent. Yield: 41.2%; dark yellow solid; mp 152–155 °C ([29], 131–134 °C); ^{1}H NMR (DMSO-d_6): δ = 2.16 (s, 3H, CH$_3$), 3.74 (t, 2H, J = 5.4 Hz, CH$_2$C$\underline{H_2}$Br), 4.21 (t, 2H, J = 5.0 Hz, C$\underline{H_2}$CH$_2$Br), 5.12 (s, 2H, CH$_2$), 5.15 (s, 2H, CH$_2$), 5.17 (s, 2H, CH$_2$), 6.77 (s, 4H, 4 × Ar-H″), 6.82 (dd, 1H, ^3J = 2.3 Hz, ^2J = 8.8 Hz, Ar-H-6), 7.11–7.14 (m, 3H, 2 × Ar-H′, Ar-H-4), 7.21 (d, 1H, ^2J = 8.8 Hz, Ar-H-7), 7.31 (d, 2H, J = 8.7 Hz, 2 × Ar-H′), 7.34–7.50 (m, 10H, 2 × Ph) ppm.

*2.1.9. Synthesis of 1-(4-(2-(azepan-1-yl)ethoxy)benzyl)-2-(4-(benzoyloxy)phenyl)-3-methyl-1H-indol-5-yl benzoate (**11**) [29]*

Hexamethylenimine (1.16 mL, 9.80 mmol) was added to a solution of compound **10** (0.650 g, 0.98 mmol) in dry tetrahydrofuran (20 mL) and the reaction mixture refluxed overnight. The solvent was evaporated under reduced pressure and the residue dissolved in ethyl acetate (30 mL) and washed with saturated aqueous $NaHCO_3$ solution (30 mL) and brine (30 mL). It was subsequently dried over Na_2SO_4, filtered and the solvent removed *in vacuo*. The crude product was purified by flash column chromatography using dichloromethane/methanol (15:1) as eluent. Yield: 64.5%; white solid; mp 98–100 °C ([29], 106–107 °C); ^{1}H NMR (DMSO-d_6): δ = 1.47–1.60 (m, 8H, 4 × azepane CH$_2$), 2.16 (s, 3H, CH$_3$), 2.59–2.70 (m, 4H, 4 × azepane CH$_2$), 2.79 (t, 2H, J = 5.6 Hz, CH$_2$C$\underline{H_2}$N), 3.93 (t, 2H, J = 5.6 Hz, C$\underline{H_2}$CH$_2$N), 5.12 (s, 2H, CH$_2$), 5.15 (s, 2H, CH$_2$), 5.16 (s, 2H, CH$_2$), 6.75 (s, 4H, 4 × Ar-H″), 6.81 (dd, 1H, ^3J = 2.4 Hz, ^2J = 8.8 Hz, Ar-H-6), 7.11–7.14 (m, 3H, 2 × Ar-H′, Ar-H-4), 7.21 (d, 1H, ^2J = 8.8 Hz, Ar-H-7), 7.30 (d, 2H, J = 8.7 Hz, 2 × Ar-H′), 7.34–7.50 (m, 10H, 2 × Ph) ppm.

*2.1.10. Synthesis of 1-(4-(2-(azepan-1-yl)ethoxy)benzyl)-2-(4-hydroxyphenyl)-3-methyl-1H-indol-5-ol (**12**) [29]*

Compound **11** (0.420 g, 0.62 mmol) was dissolved in a mixture of ethanol/tetrahydrofuran = 1:1 (25 mL). Following this, 10% Pd/C (80 mg) was added and the reaction mixture stirred under a hydrogen atmosphere at room temperature overnight. The catalyst was filtered off and the solvent removed under reduced pressure. The crude product was purified by flash column chromatography using dichloromethane/methanol (9:1) as eluent. Yield: 89.2%; light brown solid; mp 108–113.5 °C; ^{1}H NMR (DMSO-d_6): δ = 1.47–1.63 (m, 8H, 4 × azepane CH$_2$), 2.10 (s, 3H, CH$_3$), 2.63–2.81 (m, 4H, 4 × azepane CH$_2$), 2.86 (t, 2H, J = 5.6 Hz, CH$_2$C$\underline{H_2}$N), 3.97 (t, 2H, J = 5.6 Hz, C$\underline{H_2}$CH$_2$N), 5.10 (s, 2H, PhC$\underline{H_2}$N), 6.58 (dd, 1H, ^3J = 2.3 Hz, ^2J = 8.7 Hz, Ar-H-6), 6.76 (s, 4H, 4 × Ar-H″), 6.81 (d, 1H, ^3J = 2.3 Hz, Ar-H-4), 6.86 (d, 2H, J = 8.6 Hz, 2 × Ar-H′), 7.06 (d, 1H, ^2J = 8.7 Hz, Ar-H-7), 7.16 (d, 2H, J = 8.7 Hz, 2 × Ar-H′), 8.67 (s, 1H, OH), 9.64 (s, 1H, OH) ppm. Purity: 97.5%. MS (ESI+): m/z (%) = 471.3 (M+H$^+$).

*2.1.11. Synthesis of 2-(4-hydroxyphenyl)-3-methyl-1H-indol-5-ol (**13**)*

After compound **4** (0.247 g, 0.55 mmol) had been dissolved in a mixture of ethanol/tetrahydrofuran = 1:1 (15 mL), 10% Pd/C (50 mg) was added and the reaction mixture stirred under a hydrogen atmosphere at room temperature overnight. The catalyst was filtered off and the solvent removed under reduced pressure. The crude product was purified by flash column chromatography using dichloromethane/methanol (9:1) as eluent. Yield: 78.3%; brown solid; mp > 300 °C; ^{1}H NMR (DMSO-d_6): δ = 2.27 (s, 3H, CH$_3$), 6.57 (dd, 1H, ^3J = 2.1 Hz, ^2J = 8.5 Hz, Ar-H-6), 6.76 (d, 1H, ^3J = 2.1 Hz, Ar-H-4), 6.88 (d, 2H, J = 8.6 Hz, 2 × Ar-H′), 7.10 (d, 1H, ^2J = 8.5 Hz, Ar-H-7), 7.44 (d, 2H, J = 8.6 Hz, 2 × Ar-H′), 8.56 (s, 1H, OH), 9.54 (s, 1H, OH), 10.59 (s, 1H, NH) ppm. MS (ESI+): m/z (%) = 240 (100) [M+H]$^+$; HRMS (ESI+): m/z [M+H]$^+$ calcd for $C_{15}H_{14}NO_2$: 240.1025, found: 240.1025.

2.2. Materials for the GSH trapping assay

Raloxifene, haloperidol, ketoconazole, human liver microsomes, recombinant CYP3A4, CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2E1,

glutathione (GSH), NADPH, $MgCl_2$, dimethyl sulfoxide (DMSO) were from Sigma Aldrich (Deisenhofen, Germany).

2.3. Incubations with human liver microsomes or recombinant CYP450 isozymes

All incubations (total volume, 100 μL) were conducted at 37 °C for 60 min in a 50 mM phosphate buffer (pH 7.4) containing 1 mg/mL of human liver microsomes or recombinant CYP450 isozymes, 5 mM $MgCl_2$, 5 mM GSH and 1 mM NADPH. Firstly, the microsomes were suspended in a mixture of buffer, $MgCl_2$ and GSH. Raloxifene, bazedoxifene or 2-(4-hydroxyphenyl)-3-methyl-1*H*-indol-5-ol in DMSO was added to a final concentration of 50 μM. The final concentration of DMSO in the incubation media was 1%. The reaction mixture was preincubated at 37 °C for 5 min. Twenty microliters of 5 mM NADPH was added in order to initiate the reaction. The reaction was quenched by adding 25 μL of ice cold acetonitrile containing haloperidol as an internal standard. The incubation mixture was left at −20 °C for 24 h and then centrifuged at 1300*g* for 100 min at 4 °C. The supernatants were transferred into a 96-well plate for subsequent LC–MS/MS analysis. Incubations that lacked NADPH, GSH, or microsomes served as negative controls. In addition, the formation of GSH conjugates was monitored in the presence of a CYP3A4 inhibitor, ketoconazole (1 μM).

2.4. LC–MS/MS analysis of glutathione conjugates

For the purposes of metabolite profiling, the resulting supernatants were analyzed using an Agilent 6460 triple quadrupole mass spectrometer equipped with a JetStream™ interface and coupled to an Agilent 1290 Infinity UPLC (Agilent Technologies, Santa Clara, USA). The chromatographic separation was performed on a Kinetex 100 × 3.0 mm C18 column (2.6-μm particles), guarded by a 4 × 2 mm C18 mm cartridge column (Phenomenex, Torrance, USA) and kept at 50 °C. The injection volume was 1.0 μL. After each injection, the injection needle was washed for 15 s with 80% MeOH. The mobile phase A was 0.1% formic acid in water and the mobile phase B was 100% acetonitrile. The flow rate was 0.5 mL/min with the following linear gradient points (time [min], % of B): (0,10); (0.5,10); (0.8,20); (1.5,40); (1.9,60); (2.3,60); (2.5,10); (3.5,10). The ion source parameters were set as follows: drying gas temperature 275 °C, drying gas flow rate 5 L/min, nebulizer 45 PSI (3.1×10^5 Pa), sheath gas temperature 320 °C, sheath gas flow 11 L/min, capillary entrance voltage 4000 V, nozzle voltage 2000 V, delta EMV 200 V. The MS detector was operated using the negative precursor ion scan mode. The MS1 scan range was m/z 300–800 with a resolution of 0.7, while the scan time was 500 ms and the fragmentor was set at 135 V; the collision energy was 12 eV. Instrument control, data acquisition and quantification were performed using MassHunter Workstation software B.03.01 (Agilent Technologies, Torrance, USA).

3. Results

3.1. Incubation of raloxifene with HLM and with recombinant CYP3A4

While the glutathione conjugate of raloxifene was formed in the incubation with both HLM and recombinant CYP3A4 in the presence of NADPH and GSH (Fig. 2), it was not found in the control incubations without NADPH and/or GSH. In contrast to Chen et al. [9] and Yu et al. [30], who suggested the formation of three isomeric glutathione conjugates of raloxifene, only one chromatographic peak corresponding to the raloxifene-glutathione adduct was observed in the current study when searching for the precursor ions of the fragment *m/z* 272 in negative ionization mode, which is considered a typical fragment ion for glutathione-adducts [19] (Fig. 2). The presence of the precursor ion at *m/z* 777 confirmed the raloxifene-glutathione adduct (with an expected mass shift of 305). Similarly, only one glutathione conjugate of raloxifene was observed in the study carried out by Dalvie et al. [12]. In the presence of ketoconazole, the raloxifene glutathione conjugate was not formed, a consequence of the strong inhibition of CYP by ketoconazole; this observation was consistent with the results of Chang et al. [31]. Moreover, so as to demonstrate the involvement of CYP3A4 in raloxifene bioactivation, experiments on recombinant CYP3A4 were performed and the results also showed the formation of raloxifene glutathione conjugates. Based on successful formation of the raloxifene glutathione conjugate, it can be concluded that our incubation system was appropriate.

3.2. Incubation of bazedoxifene with HLM and with recombinant CYP450 isozymes

In the case of bazedoxifene, no glutathione conjugates were detected in the incubation with HLM (Fig. 3, *m/z* 272 precursor ion scan expecting a conjugate signal at *m/z* 774 resulting from mass

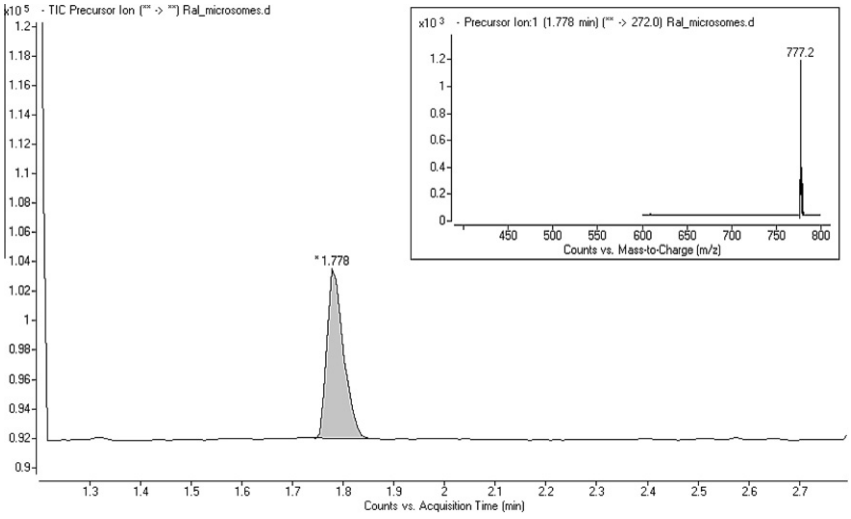


Fig. 2. The negative precursor ion scan chromatogram of the HLM-raloxifene incubation medium (searching for precursors from fragment m/z 272) with the corresponding spectrum extracted from the peak at 1.778 min that shows the molecular mass of the raloxifene glutathione conjugate peak (*m/z* 777.2).

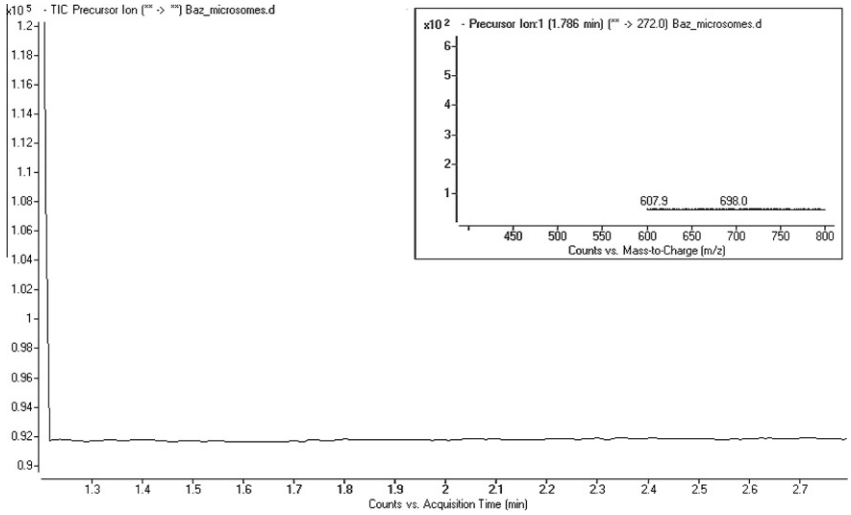


Fig. 3. Total ionic chromatogram of precursor ion scan of the bazedoxifene – HLM incubation sample (searching for precursors from fragment *m/z* 272) where no peaks for bazedoxifene glutathione conjugates were observed. The empty MS spectrum at the expected retention time (around 1.8 min) is presented in the upper right corner, which shows the absence of any GSH conjugates.

Table 1
Formation of GSH conjugates by human P450 isozymes, expressed as areas of LC–MS/MS peaks obtained from extracted precursor ion (m/z 272) scan chromatograms.

System	Bazedoxifene[a]	Compound **13**[b]
Control	–	–
CYP1A2	2012	1532,214
CYP3A4	3266	364,722
CYP2C8	3306	302,654
CYP2C9	8596[c]	779,304
CYP2C19	–	528,737
CYP2E1	8250	312,108

[a] *m/z* = 791 (470 + 305 + 16); t_r = 1.505 min.
[b] *m/z* = 543 (238 + 305); t_r = 1.749 min.
[c] In this incubation, traces of GSH conjugate of compound 13 were detected as well (corresponding area 4641).

shift 305 from the parent as was observed with raloxifene). Based on the positive results of glutathione conjugate formation of raloxifene (precursor scan *m/z* 272 giving a strong signal at *m/z* 777, Fig. 2), which validated our in vitro incubation system, the negative results of bazedoxifene glutathione conjugate formation can be considered reliable.

Bazedoxifene was also incubated with human recombinant cytochrome P450 isozymes, and the amounts of GSH conjugates formed determined (Table 1). Just as in the case of the bazedoxifene – HLM incubation sample, no GSH conjugates were detected (*m/z* 272 precursor ion scan expecting a conjugate signal at *m/z* 774 or *m/z* 491 resulting from mass shift 305 or 321, respectively). None of the selected human cytochrome P450 isozymes catalyzed the formation of reactive species of bazedoxifene, while only traces of GSH conjugates were observed in incubations with CYP2C9 and CYP2E1 (Table 1). The *N*-dealkylated metabolite of bazedoxifene (compound **13**) was detected neither after the incubation of bazedoxifene with HLM nor with recombinant P450 isozymes (molecular ion $[M+H]^+$ *m/z* 240); the only exception noted was that of a trace amount observed in CYP2C9 incubation.

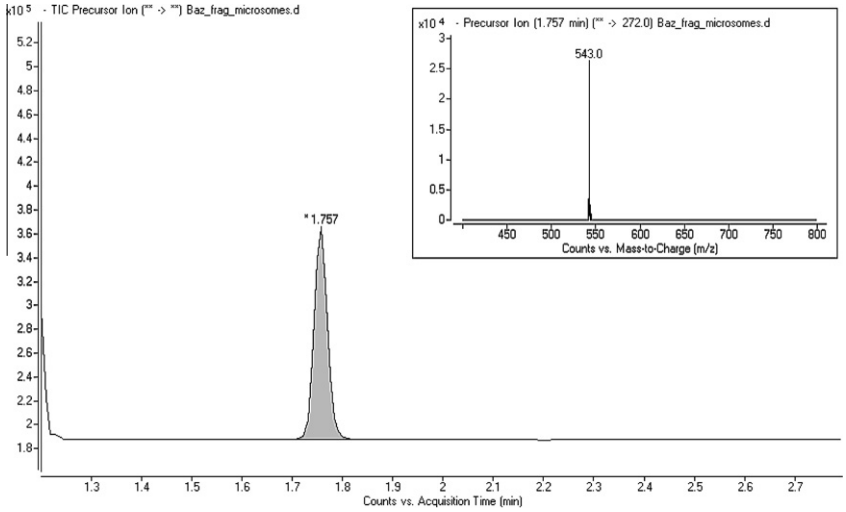


Fig. 4. The negative precursor ion scan chromatogram of compound **13** in HLM incubation medium, a survey-scan for precursors of *m/z* 272 fragment ions characteristic for glutathione conjugates. Upper right: extracted mass spectrum from peak at retention time 1.757 min, which shows the $[M-H]^-$ molecular peak of 2-(4-hydroxyphenyl)-3-methyl-1*H*-indol-5-ol-glutathione conjugate (mass shift 305 from the parent [32]).

3.3. Incubation of 2-(4-hydroxyphenyl)-3-methyl-1H-indol-5-ol (13) with HLM and recombinant CYP450 isozymes

One GSH adduct of 2-(4-hydroxyphenyl)-3-methyl-1*H*-indol-5-ol (**13**), a 5-hydroxy-3-methylindole-based fragment of bazedoxifene, was detected. The LC–MS/MS precursor ion scan of the CYP3A4 incubation medium, searching for precursors of the fragment ion *m/z* 272 in negative mode, revealed a strong peak corresponding to the GSH conjugate (*m/z* $[M-H]^-$ 543: a mass shift of 305 from the parent *m/z* 238, Fig. 4). This strongly supports the identification of a GSH adduct of compound **13**. The GSH adduct showed a characteristic loss of *m/z* 272, as evidenced by the precursor ion scan experiment. It was established that the tested bazedoxifene fragment (compound **13**) forms glutathione conjugates in the microsomal and the human recombinant CYP450 isozyme incubation systems (Table 1). CYP1A2 showed the highest activity, while the activity of other selected CYPP450s decreased in the following order CYP2C9 > CYP2C19 > CYP3A4 ≈ CYP2E1 ≈ CYP2C8.

4. Discussion

In the present study, the bioactivation potential of bazedoxifene, as a third generation SERM, was evaluated using the GSH trapping assay with HLMs and recombinant CYP450 isozymes and compared to the structurally related second generation SERM, raloxifene, and to 2-(4-hydroxyphenyl)-3-methyl-1*H*-indol-5-ol (**13**), a 5-hydroxy-3-methylindole-based structural fragment of bazedoxifene. Our results demonstrated that bazedoxifene forms only trace amounts of reactive electrophilic species in incubation with HLM or recombinant CYPP450 isozymes (Table 1) in the presence of NADPH and GSH. It is, therefore, most likely that it does not cause adverse effects by covalently binding to nucleophilic residues of proteins and/or DNA.

Bazedoxifene was synthesized, since it was not commercially available at that time. The synthesis of bazedoxifene is outlined in Scheme 1 [29]. Firstly, phenol **1** was protected with benzyl bromide to obtain ketone **2**, which was then brominated at its α position to obtain α-bromopropiophenone **3**. Indole core **4** was synthesized via a Bischler-type indole synthesis from compound **3** and 4-benzyloxyaniline hydrochloride in *N,N*-dimethylformamide using the two step – one pot procedure. The benzyl chloride **7** was prepared from 4-hydroxybenzyl alcohol (**5**) by alkylation with ethyl bromoacetate (compound **6**), followed by the nucleophilic substitution of the hydroxyl group by chlorine using thionyl chloride and a catalytic amount of *N,N*-dimethylformamide. Indole **4** was *N*-alkylated with benzyl chloride **7**, using NaH-furnished indole **8**. The side chain ester group of **8** was then reduced with $LiAlH_4$ in THF and the obtained alcohol **9** converted to bromide **10** by means of treatment with tetrabromomethane and triphenylphosphine. Nucleophilic substitution of bromine in compound **10** with hexamethylenimine yielded amine **11**, which, in the last step, was deprotected using catalytic hydrogenation to obtain bazedoxifene **12** with a percentage purity of 97.5%, as determined by HPLC analysis. 2-(4-Hydroxyphenyl)-3-methyl-1*H*-indol-5-ol (**13**), a bazedoxifene structural fragment, was synthesized by a process of the catalytic hydrogenation of compound **4**.

Previously, the most widely used LC–MS/MS method for glutathione-conjugate detection was the neutral loss scan of *m/z* 129 in positive ionization mode. Recently, the rather important discovery was made that the negative precursor ion scan mode of *m/z* 272 yields far better detection, since the background noise in negative ionization tends to be lower. Furthermore, any GSH bond linkage variations that could hinder the neutral loss of *m/z* 129, would still be detected by the precursor ion scan of *m/z* 272 [33].

The results obtained by HLM and recombinant CYP450 isozymes assays were comparable. The incubations of raloxifene showed considerable formation of the GSH-conjugate in incubations with HLM or recombinant CYP3A4, while bazedoxifene GSH-conjugates were detected only in trace amounts with HLM and six selected CYP450 isozymes (CYP1A2, CYP3A4, CYP2C8, CYP2C9, CYP2C19 and CYP2E1). The detection of raloxifene GSH-conjugates supported previously existing evidence for the potential of raloxifene bioactivation and the formation of reactive metabolites and confirmed the suitability of the incubation conditions. The presence of ketoconazole as a strong CYP3A4 inhibitor [34] caused the complete disappearance of the GSH-conjugate peak of raloxifene, indicating that CYP3A4 is an important CYP450 isoform in the formation of reactive metabolites.

Although bazedoxifene contains a potentially toxic 5-hydroxy-3-methylindole moiety, our studies showed that bazedoxifene was bioactivated to electrophilic intermediates with HLM or with six selected recombinant CYP450 isozymes only in insignificant quantities. Based on numerous reports of the bioactivation of 5-hydroxy- and 3-methylindole-based compounds, we tested the *N*-dealkylated moiety of bazedoxifene (compound **13**) in the GSH trapping assay. The GSH adduct of compound **13** was identified with HLM and with all six selected recombinant CYP450 isozymes with the aid of precursor ion scan experiments (*m/z* 272). Among the individual CYP450s present to a high degree in the pooled human liver microsomes, CYP1A2 showed the highest activity. The activity of other selected P450s decreases in the following rank order CYP2C9 > CYP2C19 > CYP3A4 ≈ CYP2E1 ≈ CYP2C8. Compound **13** is bioactivated by CYP450s to the corresponding quinoneimine intermediate or through a dehydrogenation pathway to an electrophilic 3-methyleneindolenine intermediate that is trapped with GSH. Based on these results, we can conclude that bazedoxifene requires an initial *N*-dealkylation reaction on the indole moiety, which can subsequently lead to the formation of reactive intermediates. However, an *N*-dealkylated metabolite **13** of bazedoxifene was not detected after the incubation of bazedoxifene with HLM or recombinant CYP450 isozymes (molecular ion $[M+H]^+$ *m/z* 240), with the exception of a trace amount observed in CYP2C9 incubation. The in vitro data suggest that bazedoxifene is not bioactivated with recombinant CYP450 isozymes or HLM to form reactive species, thus warranting further in vivo investigation of bazedoxifene bioactivation in order to confirm the in vitro results.

In conclusion, our results confirm that bazedoxifene, a new third generation, indole-based ER-ligand, which has been developed for use in post-menopausal osteoporosis, offers an improved safety profile relative to SERM therapies currently available. Bazedoxifene was not dehydrogenated with recombinant CYP450 isozymes or HLM to form reactive electrophilic species and is therefore unlikely to cause adverse effects by covalently binding to the nucleophilic residues of proteins and/or DNA. The results of the studies with bazedoxifene and its structural 5-hydroxy-3-methylindole-based fragment, coupled with several reports on other 3- and 5-substituted indole-containing drugs, provide additional evidence that this aromatic moiety should be used with caution in the development of new therapeutic agents. This study provides further proof that not all compounds possessing a potential structural fragment for bioactivation (structural alert) will necessarily, under bioactivation, elicit the formation of reactive species in vitro.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cbi.2012.03.001.

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