

# Inhibition of Human Sulfotransferase 2A1-Catalyzed Sulfonation of Lithocholic Acid, Glycolithocholic Acid, and Tauroolithocholic Acid by Selective Estrogen Receptor Modulators and Various Analogs and Metabolites<sup>§</sup>

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## ABSTRACT

Lithocholic acid (LCA) is a bile acid associated with adverse effects, including cholestasis, and it exists in vivo mainly as conjugates known as glyco-LCA (GLCA) and tauro-LCA (TLCA). Tamoxifen has been linked to the development of cholestasis, and it inhibits sulfotransferase 2A1 (SULT2A1)-catalyzed dehydroepiandrosterone (DHEA) sulfonation. The present study was done to characterize the sulfonation of LCA, GLCA, and TLCA and to investigate whether triphenylethylene (clomifene, tamoxifen, toremifene, ospemifene, droloxifene), benzothiophene (raloxifene, arzoxifene), tetrahydronaphthalene (lasofoxifene, nafoxidine), indole (bazedoxifene), and benzopyran (acolibifene) classes of selective estrogen receptor modulator (SERM) inhibit LCA, GLCA, and TLCA sulfonation. Human recombinant SULT2A1, but not SULT2B1b or SULT1E1, catalyzed LCA, GLCA, and TLCA sulfonation, whereas each of these enzymes catalyzed DHEA sulfonation. LCA, GLCA, and TLCA sulfonation

is catalyzed by human liver cytosol, and SULT2A1 followed the substrate inhibition model with comparable apparent  $K_m$  values ( $\leq 1 \mu\text{M}$ ). Each of the SERMs inhibited LCA, GLCA, and TLCA sulfonation with varying potency and mode of enzyme inhibition. The potency and extent of inhibition of LCA sulfonation were attenuated or increased by structural modifications to toremifene, bazedoxifene, and lasofoxifene. The inhibitory effect of raloxifene, bazedoxifene, and acolbifene on LCA sulfonation was also observed in HepG2 human hepatocellular carcinoma cells. Overall, among the SERMs investigated, bazedoxifene and raloxifene were the most effective inhibitors of LCA, GLCA, and TLCA sulfonation. These findings provide insight into the structural features of specific SERMs that contribute to their inhibition of SULT2A1-catalyzed LCA sulfonation. Inhibition of LCA, GLCA, and TLCA detoxification by a SERM may provide a biochemical basis for adverse effects associated with a SERM.

## Introduction

Bile acids, which are important endogenous signaling molecules (Đanić et al., 2018), play essential physiologic roles, including stimulation of bile flow and biliary phospholipid secretion, cholesterol elimination, and intestinal absorption of dietary fats/cholesterol, fat-soluble vitamins, and drugs (Hofmann, 1999). Disruption in homeostasis of bile acids occurs when there is dysregulation in the

synthesis/secretion, transport, or biotransformation of these endogenous substances (Li and Apte, 2015). Lithocholic acid (LCA), a secondary bile acid, is one of the most toxic bile acids (Latta et al., 1993; Song et al., 2011). It is formed by intestinal bacterial  $7\alpha$ -dehydroxylation of chenodeoxycholic acid and ursodeoxycholic acid (Hofmann, 2004). Administration of LCA to rodents results in various toxic effects such as cholestasis, liver cirrhosis, and carcinogenesis (Takikawa et al., 1991; Woolbright et al., 2014). LCA is detoxified predominantly to form lithocholic acid sulfate (LCA-S) in a sulfonation reaction catalyzed by sulfotransferase 2A1 (SULT2A1) (Huang et al., 2010; Kurogi et al., 2011). More than 95% of bile acids are found as their amidates, either as a glycine conjugate or a taurine conjugate known as glycolithocholic acid (GLCA) and tauroolithocholic acid (TLCA), respectively (Falany et al., 1994). GLCA and TLCA undergo sulfonation to form GLCA

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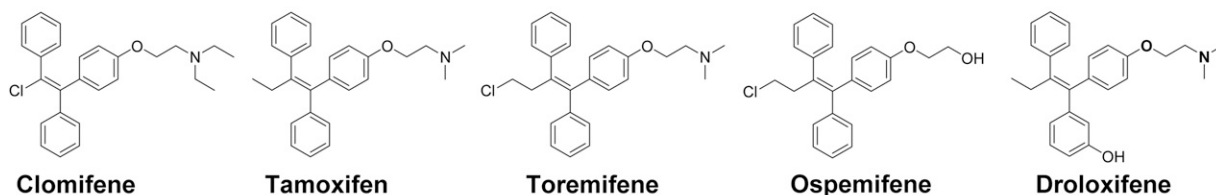
<sup>§</sup> This article has supplemental material available at [jpet.aspetjournals.org](http://jpet.aspetjournals.org).

**ABBREVIATIONS:** DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulfate; DMSO, dimethylsulfoxide; GLCA, glycolithocholic acid; GLCA-S, glycolithocholic acid sulfate; HepG2 cells, human hepatocellular carcinoma cells;  $K_i$ , inhibitory constant or equilibrium dissociation constant for the enzyme-inhibitor complex;  $K_m$ , Michaelis-Menten constant; LC, liquid chromatography; LCA, lithocholic acid; LCA-S, lithocholic acid sulfate; MIC, minimum inhibitory concentration; MS, mass spectrometry; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; SERM, selective estrogen receptor modulator; SULT, sulfotransferase; TLCA, glycolithocholic acid; TLCA-S, tauroolithocholic acid sulfate; UPLC, ultra-high-performance liquid chromatography.

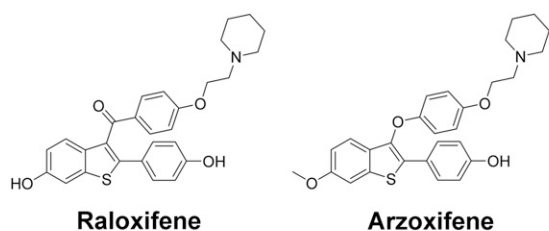
sulfate (GLCA-S) and TLCA sulfate (TLCA-S), respectively (Chen and Segel, 1985), which are more water-soluble metabolites, in a reaction catalyzed by SULT2A1 (Huang et al., 2010).

Selective estrogen receptor modulators (SERMs, Fig. 1), such as tamoxifen, act as agonists or antagonists of estrogen receptors, depending on the target tissue (Patel and Bihani, 2018). These drugs are used primarily for the treatment of

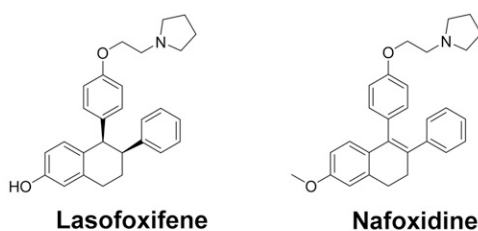
### A Triphenylethylene Class



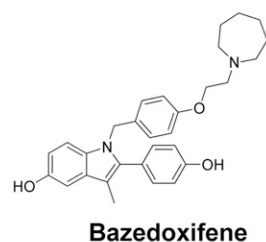
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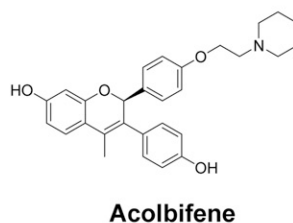
### C Tetrahydronaphthalene Class



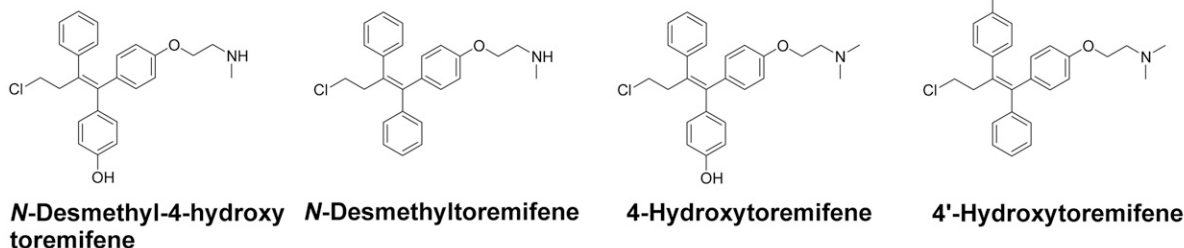
### D Indole Class



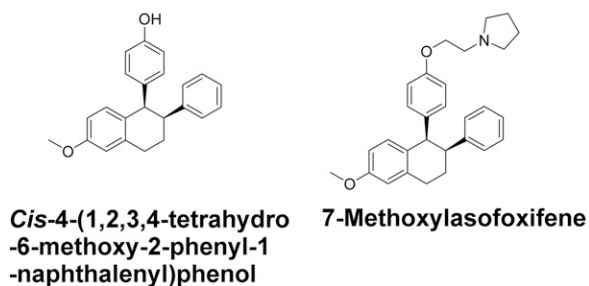
### E Benzopyran Class



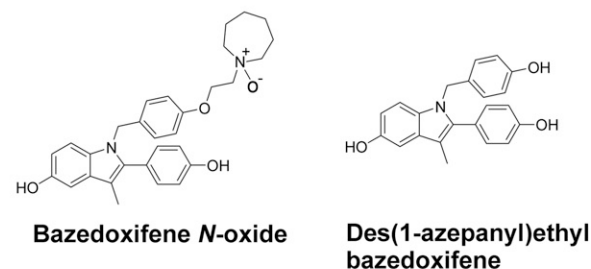
### F Toremifene Metabolites



### G Lasofoxifene Analogues



### H Bazedoxifene Metabolites / Analogues



**Fig. 1.** Chemical structures of SERMs and their metabolites/analogues. SERMs are grouped chemically into triphenylethylene (A), benzothiophene (B), tetrahydronaphthalene (C), indole (D), and benzopyran (E) classes. Also shown are the chemical structures for the metabolites and analogs of toremifene (F), lasofoxifene (G), and bazedoxifene (H).

breast cancer, prevention of osteoporosis, menopausal symptoms, and other endocrine-related conditions (Pickar et al., 2010). Drug development efforts over the years have led to the regulatory approval of third-generation SERMs, such as bazedoxifene, lasofoxifene, and ospemifene, for therapeutic use in humans (Dowers et al., 2006; DeGregorio et al., 2014; Patel and Bihani, 2018) and phase 3 clinical trials of fourth-generation SERMs, such as acolbifene (Fabian et al., 2015). Chemically, SERMs are categorized into several classes: 1) triphenylethylenes (e.g., clomifene, tamoxifen, toremifene, ospemifene, and droloxifene), 2) benzothiophenes (e.g., raloxifene, arzoxifene), 3) tetrahydronaphthalenes (e.g., lasofoxifene, nafoxidine), 4) indoles (e.g., bazedoxifene), and 5) benzopyrans (e.g., acolbifene) (Dowers et al., 2006; DeGregorio et al., 2014; Patel and Bihani, 2018). Treatment of patients with a SERM, such as tamoxifen (Mazokopakis et al., 2007) or raloxifene (Vilches et al., 1998), has been associated with the onset of cholestasis (<https://livertox.nih.gov/SelectiveEstrogenReceptorModulators.htm>). As reported previously, tamoxifen and clomifene inhibit human liver cytosolic DHEA sulfonation (Bamforth et al., 1992), an enzymatic reaction now known to be catalyzed not only by SULT2A1 (Falany et al., 1989; Yip et al., 2018) but also by SULT2B1b (Geese and Raftogianis, 2001; Meloche and Falany, 2001; Yip et al., 2018) and SULT1E1 (Falany et al., 1995; Yip et al., 2018). Collectively, these findings suggest a working hypothesis that LCA overaccumulation as a result of compromised LCA sulfonation may be a contributing factor to the development of adverse effects (e.g., cholestasis) associated with the clinical use of tamoxifen or another SERM. Whether any of the SERMs inhibit human liver cytosolic SULT2A1 and the sulfonation of LCA, GLCA, and TLCA is not known.

The overall goal of the present study was to provide a detailed biochemical understanding of the effect of individual SERMs on the sulfonation of LCA and its GLCA and TLCA conjugates. An initial series of experiments were conducted to compare the enzymatic sulfonation of LCA, GLCA, and TLCA as catalyzed by human liver cytosol and recombinant sulfotransferase enzymes. Subsequent experiments were designed to: 1) investigate the effect of triphenylethylene (e.g., clomifene, tamoxifen, toremifene, ospemifene, droloxifene), benzothiophene (e.g., raloxifene, arzoxifene), tetrahydronaphthalene (e.g., lasofoxifene, nafoxidine), indole (e.g., bazedoxifene), and benzopyran classes (e.g., acolbifene) of SERM (Fig. 1) on LCA, GLCA, and TLCA sulfonation, as catalyzed by human liver cytosol and recombinant SULT2A1; 2) elucidate the mode of inhibition of LCA sulfonation by specific SERMs; 3) identify structural features of select classes of SERMs that may contribute to the SERM inhibition of LCA sulfonation, as determined by comparing the effect of the parent drug and their respective metabolites or analogs (Fig. 1) on LCA sulfonation; and 4) determine the in situ effect of SERMs on LCA sulfonation in a cellular system.

## Materials and Methods

**Chemicals and Reagents.** LCA, cholic acid, dehydroepiandrosterone (DHEA), and dehydroepiandrosterone sulfate (DHEA-S) were purchased from Steraloids, Inc. (Newport, RI), and LCA-S disodium salt was from Santa Cruz Biotechnology, Inc. (Dallas, TX). Adenosine 3'-phosphate 5'-phosphosulfate lithium salt hydrate (PAPS), sodium tauroolithocholate, tauroolithocholic acid 3-sulfate (TLCA-S) disodium salt, amoxicillin, clomifene citrate, tamoxifen, toremifene citrate, ospemifene, raloxifene, bazedoxifene acetate, DL-dithiothreitol,

2-mercaptoethanol, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Lithocholylglycine, 3-sulfolithocholic acid (GLCA-S) disodium salt, lasofoxifene, 7-methoxylasofoxifene, nafoxidine hydrochloride, bazedoxifene-*N*-oxide, des(1-azepanyl)ethylbazedoxifene, *cis*-4-(1,2,3,4-tetrahydro-6-methoxy-2-phenyl-1-naphthalenyl)phenol, *N*-desmethyl-4-hydroxytoremifene hydrochloride, *N*-desmethyltoremifene hydrochloride, 4-hydroxytoremifene, and 4'-hydroxytoremifene were bought from Toronto Research Chemicals (Toronto, ON). Droloxifene citrate was purchased from Abcam (Cambridge, UK), and arzoxifene hydrochloride and acolbifene were from AdooQ Bioscience (Irvine, CA). Minimum essential medium/Earle's balanced salts (MEM/EBSS) culture medium (#SH30244.01), fetal bovine serum (no. SV30160.03), MEM nonessential amino acids (100×), trypsin-EDTA (0.25%), L-glutamine (200 mM), penicillin G-streptomycin (100×), and phosphate-buffered saline (pH 7.4) were of HyClone brand purchased from GE Healthcare Life Sciences (Buckinghamshire, UK). Coomassie (Bradford) Protein Assay kit (no. 23200) was bought from Thermo Fisher Scientific Inc. (Waltham, MA). All other commercially available chemicals were of analytical grade or HPLC grade.

**Cytosol and Recombinant Enzymes.** Human liver cytosol (mixed gender; pool of 150 donors; catalog no. 452115, lot #38290 (ages 18–82) and #38291 (ages 18–77), Gentest brand; 75 women and 75 men) was purchased from Corning, Inc. (Corning, NY). Human recombinant SULT2A1 (catalog no. CYP104, lot no. INT044E2B) and SULT1E1 (catalog no. CYP103, lot no. INT044E1B) enzymes and control cytosol (isolated from *Escherichia coli* host cells) were purchased from Cypex Ltd. (Dundee, Scotland, UK). Human recombinant SULT2B1b enzyme (catalog no. 6174-ST-020, lot no. DADE0616011), which contained Met-1 to Glu-311 amino acids of SULT2B1b expressed in *E. coli* host cells and contained a C-terminal 6-histidine tag, was purchased from R&D Systems, Inc. (Minneapolis, MN). This recombinant SULT2B1b enzyme has been shown to be functional and has a high enzyme activity (>10 nmol/min per milligram protein) (R&D Systems, Inc.).

**DHEA Sulfonation Assay and Quantification of DHEA-S by UPLC-MS/MS.** The DHEA sulfonation assay was conducted based on previously optimized method (Bansal and Lau, 2016b; Yip et al., 2018). Calibration standards were prepared by adding freshly prepared DHEA-S stock solutions (1–1000  $\mu$ M in DMSO) to a standard incubation to give final concentrations of 1–1000 nM DHEA-S (equivalent to 0.2–200 pmol in 200  $\mu$ l). The amount of DHEA-S and cortisol (internal standard) was quantified by ultra-high-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). The UPLC-MS/MS method was previously validated and demonstrated to be more specific, easier, safer, and faster than radiometric-based sulfotransferase enzyme assays (Bansal and Lau, 2016b).

**LCA, GLCA, and TLCA Sulfonation Assay.** The optimization of LCA sulfonation assay was performed as described previously (Bansal and Lau, 2016a). In general, each 200- $\mu$ l standard incubation mixture contained potassium phosphate buffer (100 mM; pH 7.4), MgCl<sub>2</sub> (2.5 mM), substrate (LCA, GLCA, or TLCA), and various amounts of human liver cytosol or recombinant SULT2A1, SULT2B1b, or SULT1E1 enzymes, as specified in each figure legend. The final concentration of methanol was 0.5% v/v, which did not affect the catalytic activity of SULT2A1 (Ma et al., 2003). Each incubation mixture was prewarmed for 3 minutes at 37°C in a shaking water bath. PAPS, a cofactor (20  $\mu$ M; a saturating concentration that resulted in maximum enzyme activity) (Bansal and Lau, 2016a), was added to initiate the enzyme reaction. After incubating the samples at 37°C for a specific duration, as specified in each figure legend, we terminated the reaction with 200  $\mu$ l of ice-cold acetonitrile containing cholic acid (0.1  $\mu$ M final concentration in LCA sulfonation assay or 0.3  $\mu$ M final concentration in GLCA or TLCA sulfonation assay; internal standard). Each sample was mixed and placed immediately in an ice bath. The supernatant was transferred into a 96-well microplate after centrifugation of each sample at 16,000g for 15 minutes at 4°C. UPLC-MS/MS was used for the quantification of LCA-S, GLCA-S, and TLCA-S.

**Quantification of LCA-S, GLCA-S, and TLCA-S by UPLC-MS/MS.** Calibration standards were prepared by adding freshly prepared LCA-S, GLCA-S, or TLCA-S stock solutions (1–1000  $\mu\text{M}$  in DMSO) to a standard incubation mixture to give final concentrations of 1–1000 nM LCA-S (equivalent to 0.2–200 pmol in 200  $\mu\text{L}$ ), 3–3000 nM GLCA-S (equivalent to 0.6–600 pmol in 200  $\mu\text{L}$ ), or 1–3000 nM TLCA-S (equivalent to 0.2–600 pmol in 200  $\mu\text{L}$ ). Quality-control samples were prepared in the same manner as that for the calibration standards. The final amount of GLCA-S in the low-, mid-, and high-quality control samples was 1.5, 10, and 100 pmol, respectively. The final amount of TLCA-S in the low-, mid-, and high-quality control samples was 1, 10, and 100 pmol, respectively. All the standard, quality-control, and unknown samples were centrifuged at 16,000g at 4°C for 15 minutes, and an aliquot of the supernatants was transferred to a 96-well polypropylene plate for UPLC-MS/MS analysis.

The method to quantify the amount of LCA-S was modified from a previously developed and validated UPLC-MS/MS method (Bansal and Lau, 2016a). New UPLC-MS/MS methods for the quantification of GLCA-S and TLCA-S were developed and validated. An Agilent Infinity1290 LC system (Agilent Technologies, Waldbronn, Germany) coupled to an AB Sciex Triple Quad 3500 triple-quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a TurboV ion source was used. Chromatographic separation was achieved on an ACQUITY UPLC BEH  $\text{C}_{18}$  column (2.1  $\times$  50 mm, 1.7  $\mu\text{m}$ ). The column and the autosampler compartment were maintained at 45°C and 4°C, respectively. The flow rate was 0.5 mL/min, and the sample injection volume was 5  $\mu\text{L}$ . The mobile phases were (A) 10 mM ammonium acetate and (B) methanol. The gradient conditions were optimized as follows: 20% B at 0.0–1.0 minute, linear increase from 20% to 95% B at 1.0–2.5 minutes, 95% B at 2.5–5.0 minutes, linear decrease from 95% to 20% B at 5.0–5.1 minutes, and 20% B at 5.1–6.0 minutes. The total run time was 6 minutes. The UPLC effluent was introduced directly into the mass spectrometer from 1.1 to 5.0 minutes. The mass spectrometer was operated in the negative electrospray ionization mode. Compound-dependent and ion source-dependent mass spectrometric parameters were optimized to achieve maximal ion intensities in the multiple reaction monitoring mode. Nitrogen gas was used as the curtain gas, collision gas, and ion source gas. The optimized compound-dependent MS parameters and ion source parameters for LCA-S, GLCA-S, GLCA, TLCA-S, TLCA, and cholic acid are shown in Supplemental Table S1. Data acquisition and processing were performed using Analyst software version 1.6.2 (Applied Biosystems).

**Enzyme Kinetic Analysis of LCA, GLCA, and TLCA Sulfonation.** The enzyme kinetics of LCA, GLCA, or TLCA sulfonation catalyzed by human liver cytosol and recombinant SULT2A1 enzyme were performed by conducting the sulfonation assay at various concentrations of LCA, GLCA, or TLCA. Each 200- $\mu\text{L}$  standard incubation mixture contained potassium phosphate buffer (100 mM; pH 7.4),  $\text{MgCl}_2$  (2.5 mM), human liver cytosol, or recombinant SULT2A1 enzyme, and varying concentrations of a substrate, at an amount or concentration stated in the figure legend. SigmaPlot version 12.5 Enzyme Kinetics Module (Systat Software, Inc., San Jose, CA) was used to calculate the values of apparent  $K_m$  and  $V_{\text{max}}$  by nonlinear least-squares regression analysis of the rate of enzyme activity ( $V$ ) and substrate concentration ( $S$ ) data. Various measures of goodness of fit, including Akaike information criterion, coefficient of determination ( $R^2$ ), S.D. of residuals ( $\text{Sy.x}$ ), and visual inspection of the  $V$  versus  $[S]$  progress curve were considered in the selection of the best model, including the substrate inhibition model (eq. 1):

$$V = \frac{V_{\text{max}}}{1 + K_m/S + S/K_i} \quad (1)$$

where  $V_{\text{max}}$  represents the apparent maximum reaction velocity,  $K_m$  represents the substrate concentration at which the reaction rate is half of  $V_{\text{max}}$ , and  $K_i$  represents the equilibrium dissociation constant between the substrate and the binding site of the enzyme.

**Enzyme Inhibition Experiments.** Inhibition of LCA, GLCA, or TLCA sulfonation was determined by conducting enzyme activity assay in the presence a test chemical (SERM or a metabolite/analog) or methanol (0.5% v/v; vehicle), a substrate (LCA, GLCA, or TLCA), and human liver cytosol or recombinant SULT2A1 enzyme at an amount or concentration stated in each figure legend. Amoxicillin (1 mM), which did not inhibit DHEA sulfonation in a previous study (Bamforth et al., 1992), was used as a negative control. Concentration-response experiments for each of the SERMs were conducted in the presence of varying concentrations of a test chemical or methanol (0.5% v/v; vehicle), a substrate (LCA, GLCA, or TLCA), and human liver cytosol at an amount or concentration stated in each figure legend. The  $IC_{50}$  was determined by nonlinear regression analysis (SigmaPlot 12.5) using the sigmoidal dose-response (variable slope) model (eq. 2):

$$y = \min + \frac{\max - \min}{1 + \left(\frac{x}{IC_{50}}\right)^{-Hill\text{ slope}}} \quad (2)$$

where  $\min$  is the minimum inhibitory effect,  $\max$  is the maximum inhibitory effect,  $x$  is the inhibitor concentration, and  $Hill\text{ slope}$  is the Hill coefficient.

**Enzyme Kinetic Analysis of the Inhibition of LCA Sulfonation by SERMs.** To determine the inhibition kinetics, LCA sulfonation assay was conducted in the presence varying concentrations (0.2, 0.4, 0.6, or 0.8  $\mu\text{M}$ ) of LCA and varying concentrations of a SERM, as stated in the figure legend. The apparent  $K_i$  value (equilibrium dissociation constant for the enzyme-inhibitor complex) and the mode of inhibition of each SERM were determined by nonlinear regression analysis of the rate of LCA-S formation at varying concentrations of LCA and a SERM, using equations for full and partial competitive, noncompetitive, and mixed-mode inhibition (SigmaPlot 12.5). The goodness of fit for each model was evaluated by considering Akaike information criterion,  $R^2$ , and visual inspection of the data in the Lineweaver-Burk plot. The  $K_i$  value was determined using various inhibition models, including the full competitive inhibition model (eq. 3), partial mixed inhibition model (eq. 4), full mixed inhibition model (eq. 5), and full noncompetitive inhibition model (eq. 6):

$$v = \frac{V_{\text{max}}}{1 + (K_m/S) * (1 + I/K_i)} \quad (3)$$

$$v = \frac{V_{\text{max}} * \left(\frac{1 + \beta * I / (\alpha * K_i)}{(1 + I / \alpha * K_i)}\right)}{1 + (K_m/S) * \left(\frac{1 + I / K_i}{(1 + I / (\alpha * K_i))}\right)} \quad (4)$$

$$v = \frac{V_{\text{max}}}{(K_m/S) * (1 + I/K_i) + (1 + I / \alpha * K_i)} \quad (5)$$

$$v = \frac{V_{\text{max}}}{(1 + I/K_i) * (1 + K_m/S)} \quad (6)$$

where  $S$  represents the substrate concentration,  $I$  represents the inhibitor concentration,  $V_{\text{max}}$  represents the apparent maximum reaction velocity,  $K_m$  represents the substrate concentration at which the reaction rate is half of  $V_{\text{max}}$ , and  $K_i$  represents the apparent equilibrium dissociation constant for the enzyme-inhibitor complex.

**Enzyme Inactivation Experiments.** Each 200  $\mu\text{L}$  of primary incubation mixture contained potassium phosphate buffer (100 mM; pH 7.4),  $\text{MgCl}_2$  (2.5 mM), human liver cytosol (200  $\mu\text{g}$  cytosolic protein), and a SERM (each at 10  $\mu\text{M}$ ) or methanol (0.5% v/v; vehicle). The mixture was prewarmed for 3 minutes at 37°C in a shaking water bath. Enzymatic reaction was initiated by adding PAPS (20  $\mu\text{M}$ ), and the mixture was preincubated at 37°C for 0, 30, 60, or 90 minutes. An aliquot (10  $\mu\text{L}$ ) of the primary incubation mixture was transferred to a prewarmed secondary incubation mixture (190  $\mu\text{L}$ ) containing phosphate buffer (pH 7.4),  $\text{MgCl}_2$  (2.5 mM), LCA (2.5  $\mu\text{M}$ ), and PAPS (20  $\mu\text{M}$ ). This secondary incubation mixture was incubated at 37°C for 30 minutes. The reaction was terminated with 200  $\mu\text{L}$  of ice-cold acetonitrile containing cholic acid (0.1  $\mu\text{M}$  final concentration). After

mixing thoroughly, each sample was placed immediately in an ice bath and centrifuged at 16,000g for 15 minutes at 4°C. The supernatant was then transferred to a 96-well microplate for the quantification of LCA-S using UPLC-MS/MS.

**Cell Culture.** HepG2 human hepatocellular carcinoma cells (ATCC HB-8065) were purchased from American Type Culture Collection (ATCC) (Manassas, VA) and authenticated by ATCC. HepG2 cells were cultured in MEM/EBSS culture medium supplemented with MEM nonessential amino acids (1×), 2 mM L-glutamine, 100 U/ml penicillin G, 100 µg/ml streptomycin, and 10% v/v fetal bovine serum. HepG2 cells were cultured as described previously (Seow and Lau, 2017). The passage number of HepG2 cells was kept between 10 and 15.

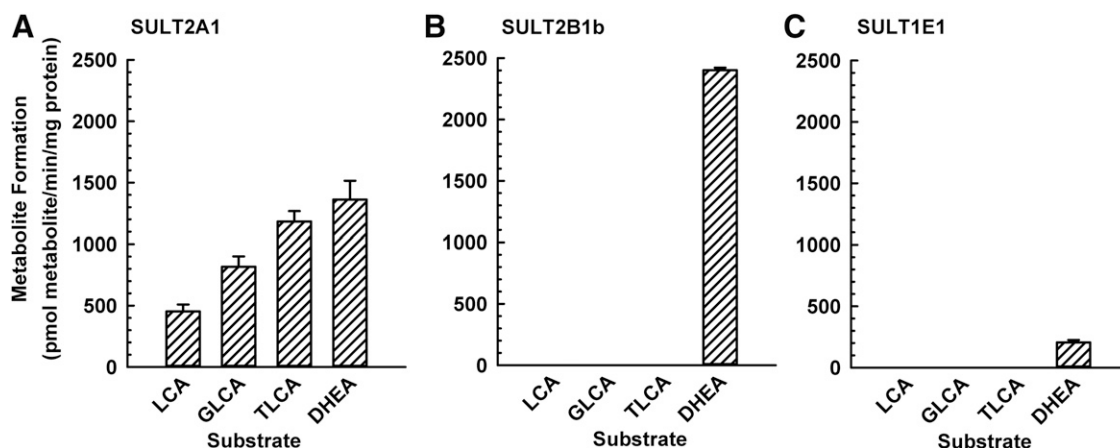
**In Situ Lithocholic Sulfonation Assay in HepG2 Cells.** In the first experimental design, cultured HepG2 cells were seeded onto 12-well plates at a cell density of 200,000 cells per well. At 96 hours after plating, HepG2 cells were washed with phosphate-buffered saline (pH 7.4). To each well, 200 µl of phosphate-buffered saline containing substrate (0.5 µM LCA) and a SERM (each at 10 µM), substrate (0.5 µM LCA) and amoxicillin (1 mM), substrate (0.5 µM LCA), and DMSO (0.1% v/v; vehicle) or DMSO alone (0.1% v/v; vehicle) was added. The cells were incubated for 15 minutes at 37°C in a humidified incubator with 95% air and 5% carbon dioxide. At the end of the incubation period, 160 µl of the incubation mixture was collected from each well and transferred into a microcentrifuge tube. The remaining incubation mixture in each well was aspirated and washed with phosphate-buffered saline. To each well, 200 µl of a lysis buffer (1% v/v Triton X-100 and 20 mM EDTA in phosphate-buffered saline) was then added. The content in each plate was mixed on a plate shaker for 1 hour to ensure complete cell lysis. At the end of cell lysis, 160 µl of the cell lysates was collected from each well and transferred into a microcentrifuge tube.

In the second experimental design, cultured HepG2 cells were seeded onto 12-well plates at a cell density of 400,000 cells per well. At 48 hours after plating, cells were treated with 1 ml of culture medium (with 5% fetal bovine serum) containing substrate (0.5 µM LCA) and a SERM (each at 10 µM), substrate (0.5 µM LCA), and amoxicillin (1 mM), substrate (0.5 µM LCA) and DMSO (0.1% v/v; vehicle) or DMSO alone (0.1% v/v; vehicle). At 24 hours after the chemical treatment period, 160 µl of the culture medium was collected from each well and transferred into a microcentrifuge tube. The remaining culture medium was aspirated, and cells were washed with phosphate-buffered saline. The lysates were prepared as described above.

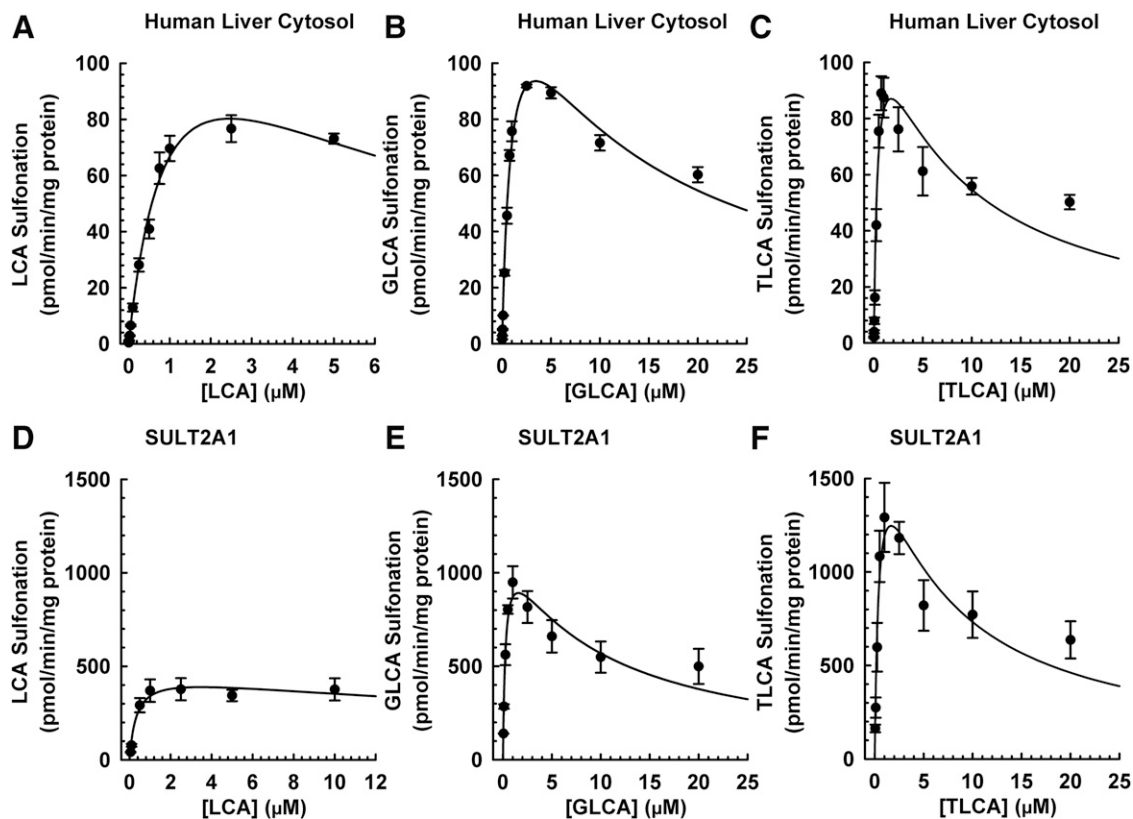
In the third experimental design, cultured HepG2 cells were seeded onto 12-well plates at a cell density of 400,000 cells per well. At 48 hours after plating, cells were treated with 1 ml of culture medium (with 5% fetal bovine serum) containing substrate (0.5 µM LCA) and a SERM (each at 10 µM), substrate (0.5 µM LCA) and amoxicillin (1 mM), substrate (0.5 µM LCA) and DMSO (0.1% v/v; vehicle), or DMSO alone (0.1% v/v; vehicle) for 24 hours. At the end of the treatment period, 200 µl of phosphate-buffered saline containing substrate (0.5 µM LCA) and a SERM (each at 10 µM), substrate (0.5 µM LCA) and amoxicillin (1 mM), substrate (0.5 µM LCA) and DMSO (0.1% v/v; vehicle), or DMSO alone (0.1% v/v; vehicle) was added. The cells were incubated for 15 minutes at 37°C in a humidified incubator with 95% air and 5% carbon dioxide. At the end of the incubation period, 160 µl of the incubation mixture was collected from each well and transferred into a microcentrifuge tube. The remaining incubation mixture in each well was aspirated and washed with phosphate-buffered saline. The lysates were prepared as described above.

To each tube of incubation mixture, culture medium, or cell lysates, 80 µl of acetonitrile containing 0.2 µM cholic acid (internal standard; final concentration of 0.1 µM in a total sample volume of 240 µl) was added and stored at -20°C before sample analysis. To prepare the calibration standards, LCA-S was serially diluted with DMSO (1–1000 µM) and further diluted by 1000× with phosphate-buffered saline, and LCA substrate (0.5 µM) was added. The final DMSO concentration was 0.2% v/v. A 160 µl of LCA-S standard solution was mixed with 80 µl of acetonitrile containing 0.2 µM cholic acid (internal standard; final concentration of 0.1 µM in a total sample volume of 240 µl). The final concentration of LCA-S was 1–1000 nM (equivalent to 0.16–160 pmol in 160 µl). The standard and sample mixtures were centrifuged at 16,000g for 15 minutes at 4°C. The supernatants were transferred into a 96-well microplate for analysis by the UPLC-MS/MS method as described herein already.

**Total Protein Quantification Assay.** At the end of the treatment/incubation period and cell lysis, the total amount of cellular protein in each well was quantified using the Coomassie (Bradford) Protein Assay kit (Thermo Fisher Scientific Inc.), according to the manufacturer's protocol. The cell lysate samples were diluted 10× using phosphate-buffered saline. The calibration curve was constructed using bovine serum albumin diluted with 10% v/v lysis buffer in phosphate-buffered saline (solvent composition similar that of the samples) to give a final concentration of 25–1000 µg/ml. A 5-µl aliquot of each sample or standard was transferred into a 96-well clear microplate, and 250 µl of the Coomassie reagent was added to each well. The content in each plate was mixed and incubated at room



**Fig. 2.** Selectivity of human recombinant SULT2A1, SULT2B1b, and SULT1E1 enzymes in LCA, GLCA, TLCA, and DHEA sulfonation. (A) Human recombinant SULT2A1 (2.5 µg of protein for GLCA, TLCA, DHEA; 5 µg of protein for LCA) was incubated with LCA (2.5 µM), GLCA (2.5 µM), TLCA (2.5 µM), or DHEA (2.5 µM) at 37°C for 30 minutes. (B) Human recombinant SULT2B1b (0.2 µg of protein) was incubated with LCA (0.5 µM), GLCA (0.5 µM), TLCA (0.5 µM), or DHEA (0.5 µM) at 37°C for 45 minutes. (C) Human recombinant SULT1E1 (5 µg of protein) was incubated with LCA (2.5 µM), GLCA (1 µM), TLCA (1 µM), or DHEA (2.5 µM) at 37°C for 30 minutes. Data are expressed as mean ± S.E.M. of three to five independent experiments conducted in duplicate.



**Fig. 3.** LCA, GLCA, and TLCA sulfonation catalyzed by human liver cytosol and human recombinant SULT2A1 at various concentrations of substrates. Human liver cytosol [80  $\mu$ g protein (A), 60  $\mu$ g protein (B), or 40  $\mu$ g protein (C)] was incubated with varying concentrations of LCA (0.0025–5  $\mu$ M) (A), GLCA (0.01–20  $\mu$ M) (B), or TLCA (0.01–20  $\mu$ M) (C) at 37°C for 15 minutes (A) or 20 minutes (B and C). Human recombinant SULT2A1 [5  $\mu$ g of protein (D) or 2.5  $\mu$ g of protein (E and F)] was incubated with varying concentrations of LCA (0.05–10  $\mu$ M) (D), GLCA (0.05–20  $\mu$ M) (E), or TLCA (0.05–20  $\mu$ M) (F) at 37°C for 30 minutes. Data are expressed as mean  $\pm$  S.E.M. of three to four independent experiments conducted in duplicate. DHEA sulfonation catalyzed by human liver cytosol and human recombinant SULT2A1 was shown in our previous study (Yip et al., 2018).

temperature for 10 minutes. The absorbance was measured at 600 nM using a plate reader (GloMax Explorer; Promega Corporation, Madison, WI). The net absorbance was calculated by subtracting the absorbance of blank sample (diluent) from that of the samples/standard sample. The amount of total cellular protein was calculated based on the calibration curve freshly prepared in each experiment.

**Statistical Analysis.** Data were analyzed by one-way or two-way analysis of variance and, where appropriate, was followed by the Student-Newman-Keuls post hoc test (SigmaPlot 12.5). The level of statistical significance was set a priori at  $P < 0.05$ .

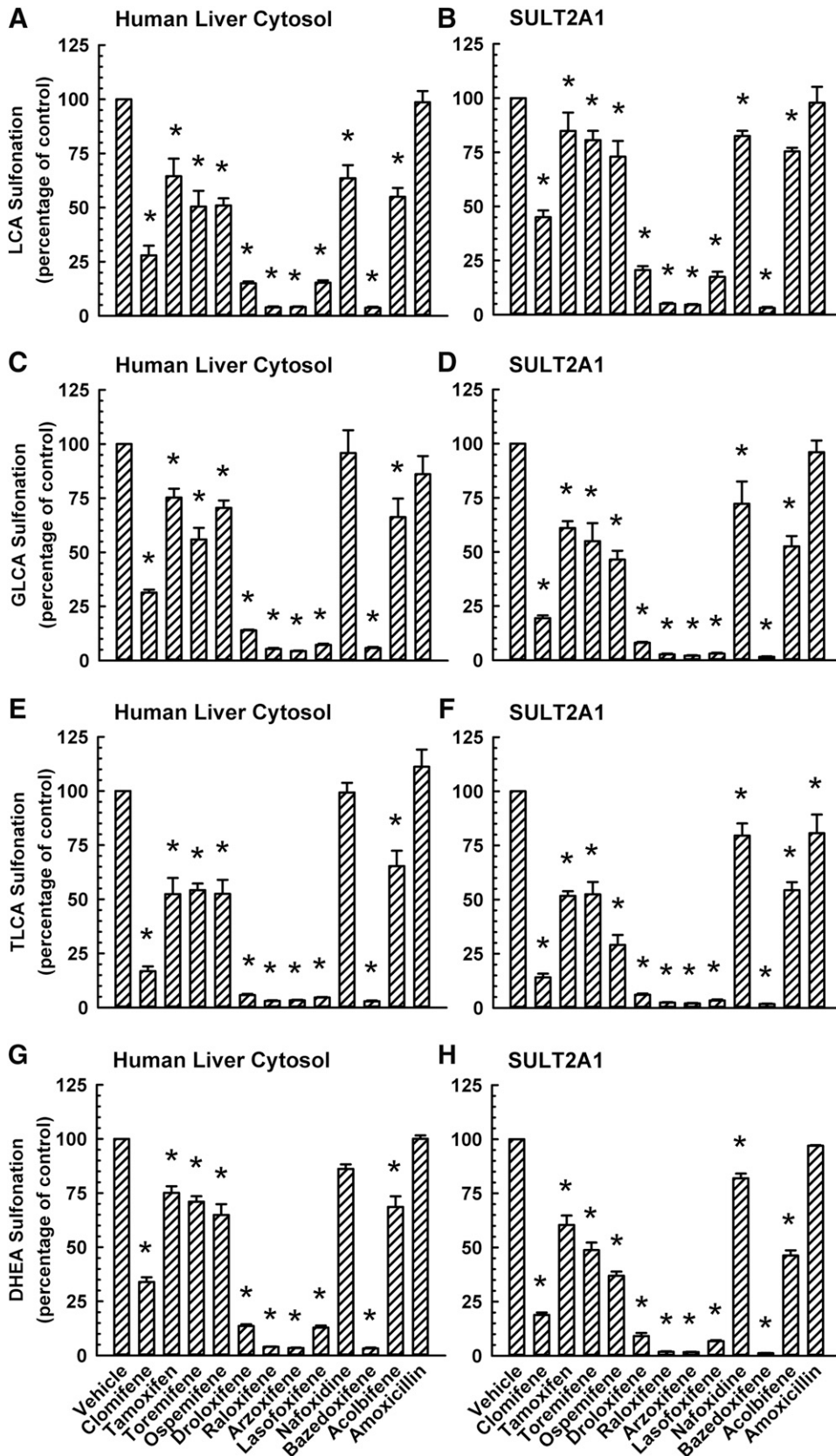
Results

**Optimization of the LCA, GLCA, TLCA, and DHEA Sulfonation Assays.** LCA sulfonation assay in human liver cytosol was optimized previously (Bansal and Lau, 2016a). LCA and GLCA sulfonation increased linearly up to 100  $\mu$ g of

cytosolic protein, whereas TLCA sulfonation increased linearly up to 80  $\mu$ g of cytosolic protein (Supplemental Fig. S1, A–C). LCA, GLCA, and TLCA sulfonation catalyzed by recombinant SULT2A1 was linear up to 5  $\mu$ g of enzyme (Supplemental Fig. S1, D–F). LCA sulfonation catalyzed by human liver cytosol was linear up to 45 minutes, whereas GLCA and TLCA sulfonation was linear up to 30 minutes (Supplemental Fig. S2, A–C). LCA and GLCA sulfonation catalyzed by SULT2A1 increased linearly up to 45 minutes, whereas TLCA sulfonation increased linearly up to 60 minutes (Supplemental Fig. S2, D–F). DHEA sulfonation assay in human liver cytosol and human recombinant SULT2A1 was performed as described in our previous study (Yip et al., 2018). Subsequent experiments were conducted using enzymatic assay conditions that were linear with respect to amount of enzyme and incubation time.

**TABLE 1**  
Enzyme kinetic analysis of lithocholic acid (LCA), glycolithocholic acid (GLCA), and lithocholic acid (TLCA) sulfonation catalyzed by human liver cytosol and recombinant sulfotransferase 2A1 (SULT2A1)  
Data are expressed as mean  $\pm$  S.E.M. of three to five independent experiments conducted in duplicate.

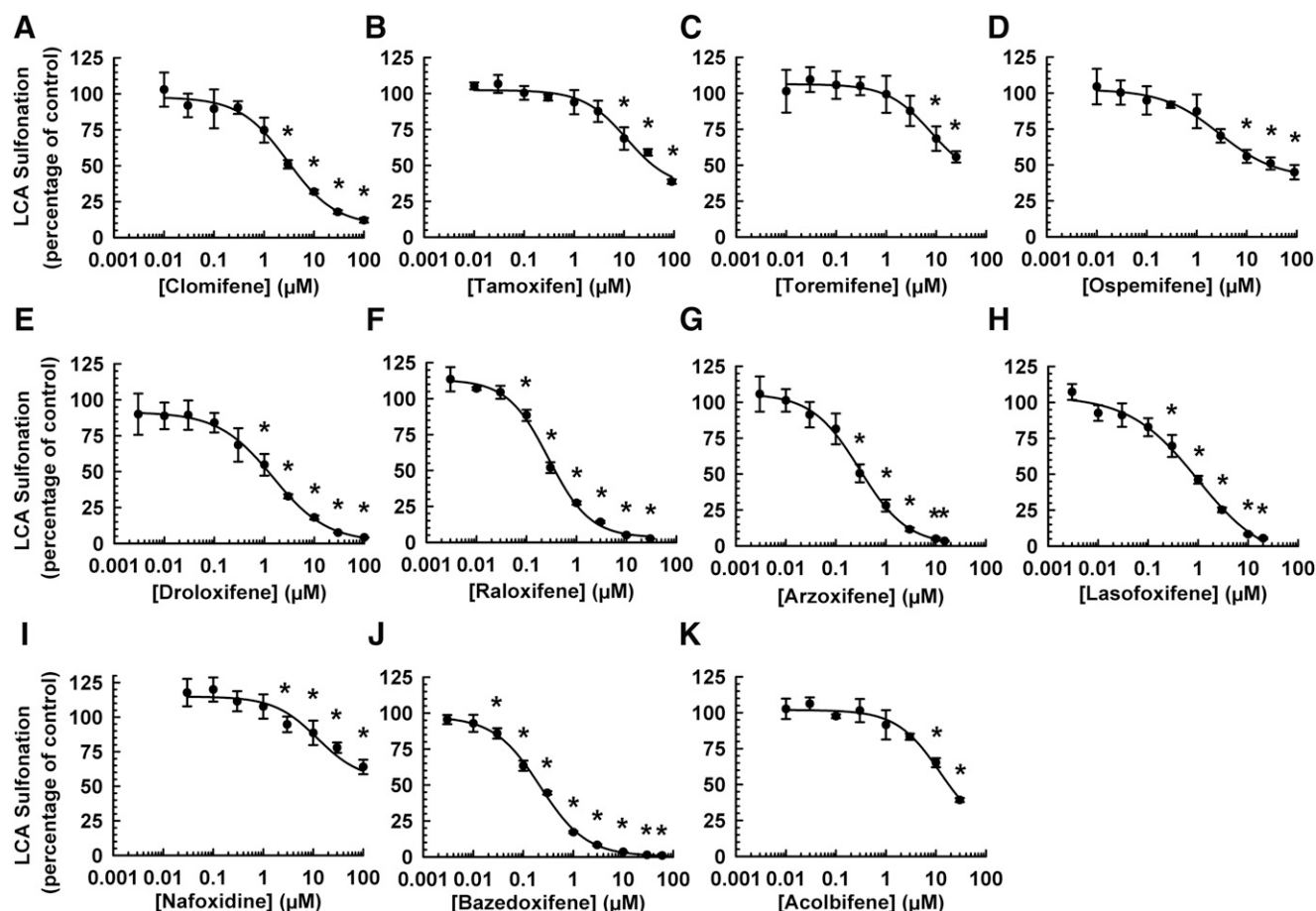
Substrate	Enzyme	$V_{max}$ (pmol/min per milligram protein)	Apparent $K_m$ ( $\mu$ M)	Apparent $K_i$ ( $\mu$ M)	$V_{max}/\text{Apparent } K_m$ ( $\mu$ l/min per milligram protein)	Enzyme Kinetics Model
LCA	Human liver cytosol	156 $\pm$ 15	1.1 $\pm$ 0.2	5.7 $\pm$ 0.9	140 $\pm$ 7	Substrate inhibition
GLCA	Human liver cytosol	148 $\pm$ 4	0.99 $\pm$ 0.07	12.3 $\pm$ 1.4	151 $\pm$ 10	Substrate inhibition
TLCA	Human liver cytosol	128 $\pm$ 15	0.40 $\pm$ 0.04	8.1 $\pm$ 1.0	320 $\pm$ 29	Substrate inhibition
LCA	Human recombinant SULT2A1	466 $\pm$ 69	0.34 $\pm$ 0.02	39.1 $\pm$ 9.6	1381 $\pm$ 185	Substrate inhibition
GLCA	Human recombinant SULT2A1	1207 $\pm$ 47	0.27 $\pm$ 0.01	9.6 $\pm$ 2.7	4467 $\pm$ 285	Substrate inhibition
TLCA	Human recombinant SULT2A1	1943 $\pm$ 100	0.48 $\pm$ 0.08	6.4 $\pm$ 1.4	4418 $\pm$ 1035	Substrate inhibition



**Fig. 4.** Effect of SERMs on human liver cytosolic and SULT2A1-catalyzed LCA, GLCA, TLCA, and DHEA sulfonation. Human liver cytosol [80  $\mu$ g protein (A, G), 60  $\mu$ g of protein (C), 40  $\mu$ g of protein (E)] or recombinant SULT2A1 [5  $\mu$ g of protein (B, H) or 2.5  $\mu$ g of protein (D, F)] was incubated with LCA [0.75  $\mu$ M for (A) or 0.5  $\mu$ M for (B)], GLCA [0.75  $\mu$ M for (C) or 0.25  $\mu$ M for (D)], TLCA [0.5  $\mu$ M for (E), or 0.25  $\mu$ M for (F)], or DHEA (0.25  $\mu$ M (G, H) and together with a SERM (clomifene, tamoxifen, toremifene, ospemifene, droloxifene, raloxifene, arzoxifene, lasofoxifene, nafoxidine, bazedoxifene, or acolbifene; 10  $\mu$ M each), amoxicillin (1 mM; negative control), or vehicle (0.5% v/v methanol) at 37°C for 15 minutes (A, G), 20 minutes (B and C), or 30 minutes (D–F and H). Data are expressed as percentage of sulfonation in the vehicle-treated control group and expressed as mean  $\pm$  S.E.M. for three to four independent experiments conducted in duplicate. \*Significantly different from the vehicle-treated control group ( $P < 0.05$ ). The rate of reaction in the vehicle-treated control group was  $103 \pm 4$  pmol/min per milligram of protein (A),  $536 \pm 45$  pmol/min per milligram of protein (B),  $49 \pm 6$  pmol/min per milligram of protein (C),  $360 \pm 59$  pmol/min per milligram of protein (D),  $41 \pm 4$  pmol/min per milligram of protein (E),  $304 \pm 34$  pmol/min per milligram of protein (F),  $124 \pm 13$  pmol/min per milligram of protein (G), and  $1520 \pm 68$  pmol/min per milligram of protein (H).

**Selectivity among SULT2A1, SULT2B1b, and SULT1E1 in LCA, GLCA, TLCA, and DHEA Sulfonation.** DHEA, which is a prototypical substrate of SULT2A1 (Falany

et al., 1989), is also metabolized by SULT2B1 (Geese and Raftogianis, 2001; Meloche and Falany, 2001; Yip et al., 2018) and SULT1E1 (Falany et al., 1995; Yip et al., 2018). Therefore,



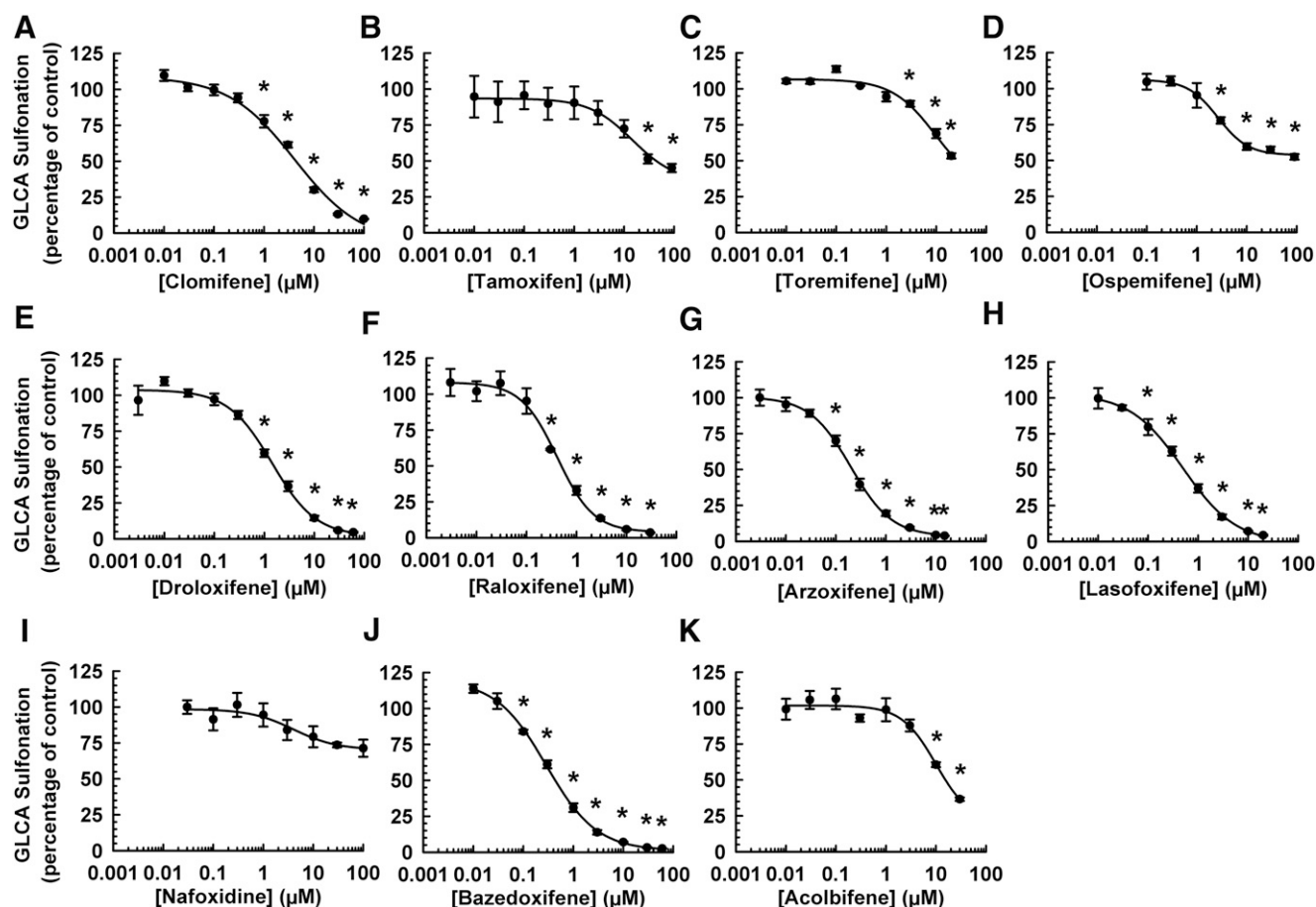
**Fig. 5.** Concentration-response relationship in the inhibitory effect of SERMs on human liver cytosolic LCA sulfonation. Human liver cytosol (80  $\mu$ g of protein) was incubated with LCA (0.75  $\mu$ M; 0.25% v/v methanol) and with varying concentrations of a SERM (clomifene (0.01–100  $\mu$ M) (A), tamoxifen (0.01–90  $\mu$ M) (B), toremifene (0.01–25  $\mu$ M) (C), ospemifene (0.01–90  $\mu$ M) (D), droloxifene (0.003–100  $\mu$ M) (E), raloxifene (0.00330  $\mu$ M) (F), arzoxifene (0.003–15  $\mu$ M) (G), lasofoxifene (0.01–20  $\mu$ M) (H), nafoxidine (0.03–200  $\mu$ M) (I), bazedoxifene (0.01–60  $\mu$ M) (J), acolbifene (0.01–30  $\mu$ M) (K), or vehicle (0.25% v/v methanol) at 37°C for 15 minute. Data are expressed as percentage of LCA sulfonation in the vehicle-treated control group and expressed as mean  $\pm$  S.E.M. for three to four independent experiments conducted in duplicate. \*Significantly different from the vehicle-treated control group ( $P < 0.05$ ).

we determined whether LCA, GLCA, and TLCA are also metabolized by these sulfotransferase enzymes. As shown in Fig. 2A, human recombinant SULT2A1 catalyzed LCA, GLCA, TLCA, and DHEA sulfonation. By comparison, SULT2B1b (Fig. 2B) and SULT1E1 (Fig. 2C) catalyzed DHEA, but not LCA, GLCA, or TLCA sulfonation, because a metabolite peak was obtained for DHEA-S but not for LCA-S, GLCA-S, or TLCA-S, as analyzed by UPLC-MS/MS. LCA-S, GLCA-S, and TLCA-S metabolite were not detected, even when the amount of enzyme, incubation time, and substrate concentration were increased (data not shown).

**Enzyme Kinetics of LCA, GLCA, TLCA, and DHEA Sulfonation Catalyzed by Human Liver Cytosol and Recombinant SULT2A1.** To compare the enzyme kinetics of LCA, GLCA, and TLCA sulfonation, human liver cytosol or SULT2A1 was incubated with varying concentrations of the respective substrate. LCA, GLCA, and TLCA sulfonation catalyzed either by human liver cytosol (Fig. 3, A–C) or human recombinant SULT2A1 (Fig. 3, D–F) exhibited substrate inhibition kinetics. The apparent  $K_m$ ,  $V_{max}$ , and ratio of  $V_{max}$ /apparent  $K_m$  values of LCA, GLCA, and TLCA sulfonation are shown in Table 1. Human liver cytosolic LCA and GLCA sulfonation yielded comparable apparent  $K_m$  and

ratio of  $V_{max}$ /apparent  $K_m$ , but smaller apparent  $K_m$  and greater ratio of  $V_{max}$ /apparent  $K_m$ . SULT2A1-catalyzed LCA, GLCA, and TLCA yielded comparable apparent  $K_m$ , but the ratio of  $V_{max}$ /apparent  $K_m$  for LCA sulfonation was smaller than that for GLCA or TLCA sulfonation. The enzyme kinetics of DHEA sulfonation catalyzed by human liver cytosol and SULT2A1 was reported in our previous study (Yip et al., 2018).

**Effect of SERMs on Human Liver Cytosol- and SULT2A1-Catalyzed LCA, GLCA, TLCA, and DHEA Sulfonation.** To determine whether the various structural classes of SERMs (Fig. 1) inhibit LCA, GLCA, and TLCA sulfonation, human liver cytosol or recombinant SULT2A1 was incubated with a substrate (0.75  $\mu$ M LCA, 0.75  $\mu$ M GLCA, or 0.5  $\mu$ M TLCA for human liver cytosol; 0.5  $\mu$ M LCA, 0.25  $\mu$ M GLCA, or 0.25  $\mu$ M TLCA for SULT2A1) and a SERM (10  $\mu$ M). SERMs in the triphenylethylene class (clomifene, tamoxifen, toremifene, ospemifene, droloxifene), benzothienopyran class (raloxifene, arzoxifene), naphthalene class (lasofoxifene, nafoxidine), indole class (bazedoxifene), and benzopyran class (acolbifene) inhibited human liver cytosol- and SULT2A1-catalyzed LCA (Fig. 4, A and B), GLCA (Fig. 4, C and D), and TLCA (Fig. 4, E and F) sulfonation, except nafoxidine did not



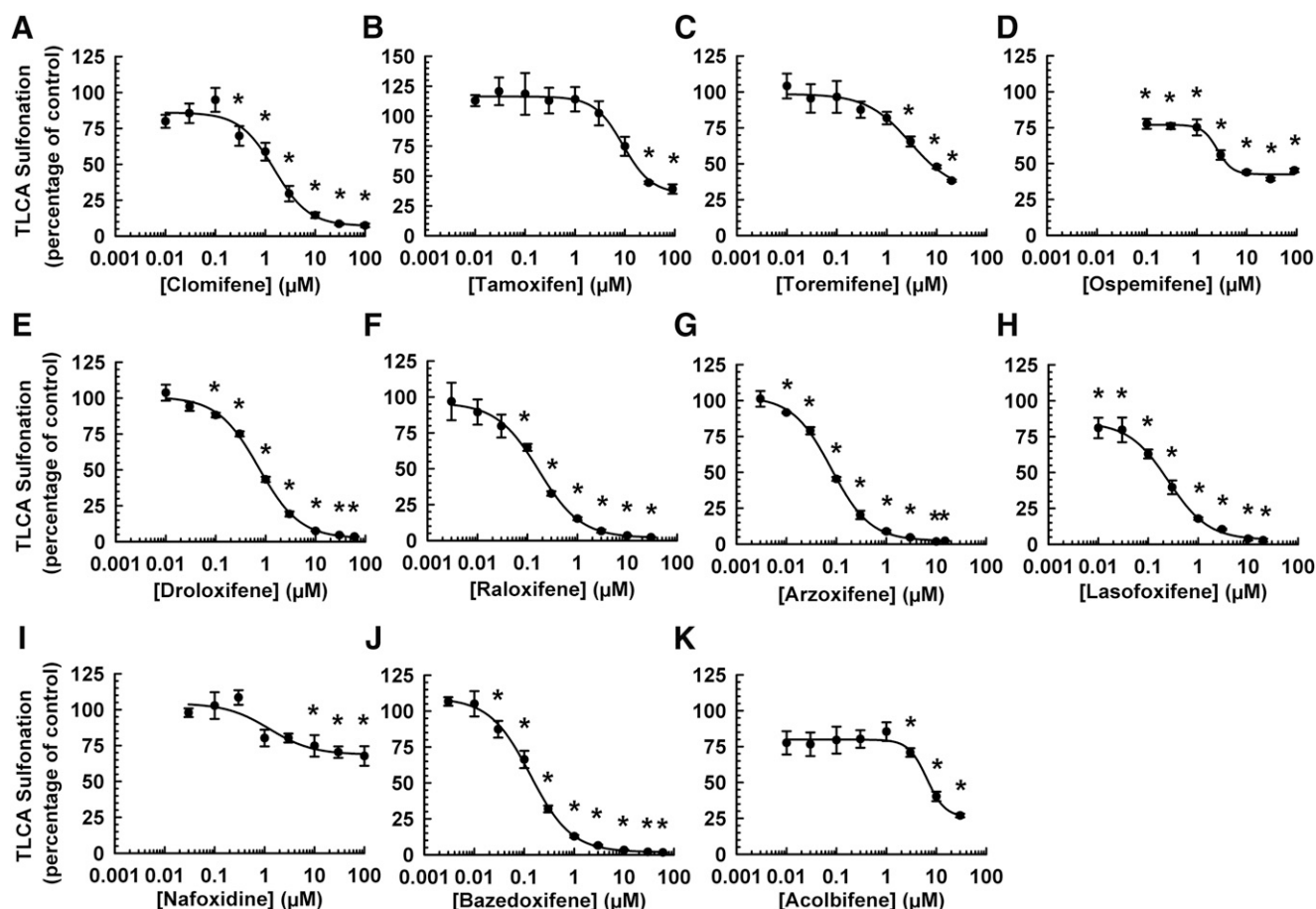
**Fig. 6.** Concentration-response relationship in the inhibitory effect of SERMs on human liver cytosolic GLCA sulfonation. Human liver cytosol (60  $\mu$ g protein) was incubated with GLCA (0.75  $\mu$ M; 0.25% v/v methanol) and with varying concentrations of a SERM [clomifene (0.01–100  $\mu$ M) (A), tamoxifen (0.01–90  $\mu$ M) (B), toremifene (0.01–20  $\mu$ M) (C), ospemifene (0.01–90  $\mu$ M) (D), droloxifene (0.003–60  $\mu$ M) (E), raloxifene (0.003–30  $\mu$ M) (F), arzoxifene (0.003–15  $\mu$ M) (G), lasofoxifene (0.01–20  $\mu$ M) (H), nafoxidine (0.03–100  $\mu$ M) (I), bazedoxifene (0.01–60  $\mu$ M) (J), acolbifene (0.01–30  $\mu$ M) (K)], or vehicle (0.25% v/v methanol) at 37°C for 20 minutes. Data are expressed as percentage of GLCA sulfonation in the vehicle-treated control group and expressed as mean  $\pm$  S.E.M. for three independent experiments conducted in duplicate. \*Significantly different from the vehicle-treated control group ( $P < 0.05$ ).

inhibit human liver cytosolic GLCA (Fig. 4C) or TLCA (Fig. 4E) sulfonation. In human liver cytosol, raloxifene, arzoxifene, and bazedoxifene inhibited LCA, GLCA, or TLCA sulfonation by more than 95%, and lasofoxifene and droloxifene inhibited it by more than 80% (Fig. 4, A, C, and E). The other SERMs, namely, clomifene, tamoxifen, toremifene, ospemifene, nafoxidine, and acolbifene inhibited LCA, GLCA, and TLCA sulfonation to a lesser extent. When tested with human recombinant SULT2A1 (Fig. 4, B, D, and F), the pattern of inhibition of LCA, GLCA, and TLCA sulfonation by each of the SERMs was similar to the results obtained with human liver cytosol. The inhibition of SULT2A1-catalyzed TLCA sulfonation by each of the SERMs investigated was greater than that on LCA or GLCA sulfonation. Comparatively, DHEA sulfonation was also inhibited by SERMs in a pattern similar to that for LCA, GLCA, and TLCA (Fig. 4, G and H). Amoxicillin, at a concentration of 1 mM and included in the present study as a negative control in the inhibition of DHEA sulfonation experiment (Bamforth et al., 1992), had little or no effect on the four enzymatic reactions catalyzed by human liver cytosol or SULT2A1 (Fig. 4).

**Concentration-Response Relationship in the Inhibition of Human Liver Cytosolic LCA, GLCA, and TLCA Sulfonation by SERMs.** Concentration-response experiment was performed to determine the  $IC_{50}$  and minimum

inhibitory concentration (MIC) of each SERM in inhibiting human liver cytosolic LCA (Fig. 5), GLCA (Fig. 6), and TLCA (Fig. 7) sulfonation. Among the SERMs tested, raloxifene (benzothiophene class), arzoxifene (benzothiophene class), lasofoxifene (naphthalene class), and bazedoxifene (indole class) were the most potent inhibitors of LCA, GLCA, and TLCA sulfonation with submicromolar  $IC_{50}$  and MIC values (Table 2). Among the triphenylethylene class of SERMs (clomifene, tamoxifen, toremifene, ospemifene, droloxifene), droloxifene was the most potent inhibitor of human liver cytosolic LCA, GLCA, and TLCA sulfonation, whereas tamoxifen was consistently the least potent inhibitor of LCA, GLCA, and TLCA sulfonation. In contrast to lasofoxifene, nafoxidine, which is a structural analog of lasofoxifene, has an  $IC_{50}$  value that was 6- to 13-fold greater than that of lasofoxifene and an MIC of 10–100  $\mu$ M. Comparatively, acolbifene, a benzopyran class of SERM, was a weak inhibitor of LCA, GLCA, and TLCA sulfonation.

**Mode of Inhibition of Human Liver Cytosolic LCA Sulfonation by Specific SERMs.** To elucidate the apparent  $K_i$  and mode of inhibition of human liver cytosol-catalyzed LCA sulfonation by specific SERMs (i.e., those that yielded more than 50% inhibition at the highest soluble concentration), enzyme kinetics experiments were performed with four



**Fig. 7.** Concentration-response relationship in the inhibitory effect of SERMs on human liver cytosolic TLCA sulfonation. Human liver cytosol (40  $\mu$ g of protein) was incubated with TLCA (0.5  $\mu$ M; 0.25% v/v methanol) and with varying concentrations of a SERM [clomifene (0.01–100  $\mu$ M) (A), tamoxifen (0.01–90  $\mu$ M) (B), toremifene (0.01–20  $\mu$ M) (C), ospemifene (0.01–90  $\mu$ M) (D), droloxifene (0.003–60  $\mu$ M) (E), raloxifene (0.003–30  $\mu$ M) (F), arzoxifene (0.003–15  $\mu$ M) (G), lasofoxifene (0.01–20  $\mu$ M) (H), nafoxidine (0.03–100  $\mu$ M) (I), bazedoxifene (0.01–60  $\mu$ M) (J), acolbifene (0.01–30  $\mu$ M) (K)], or vehicle (0.25% v/v methanol) at 37°C for 20 minutes. Data are expressed as percentage of TLCA sulfonation in the vehicle-treated control group and expressed as mean  $\pm$  S.E.M. for three independent experiments conducted in duplicate. \*Significantly different from the vehicle-treated control group ( $P < 0.05$ ).

or five concentrations of each inhibitor (selected from the linear range in the respective concentration-response curve, Figs. 5–7) and four concentrations of LCA. Lineweaver-Burk plot and nonlinear least-squares regression analyses of the enzyme kinetic data indicated that mode of inhibition of human liver cytosolic LCA sulfonation was full competitive

inhibition (clomifene, Fig. 8A), partial mixed inhibition (droloxifene, Fig. 8B), full mixed inhibition (arzoxifene, Fig. 8D), or full noncompetitive inhibition (raloxifene, Fig. 8C; lasofoxifene, Fig. 8E; and bazedoxifene, Fig. 8F). As shown in Table 3, the calculated apparent  $K_i$  values for bazedoxifene ( $0.2 \pm 0.03$   $\mu$ M), raloxifene ( $0.5 \pm 0.04$   $\mu$ M),

**TABLE 2**

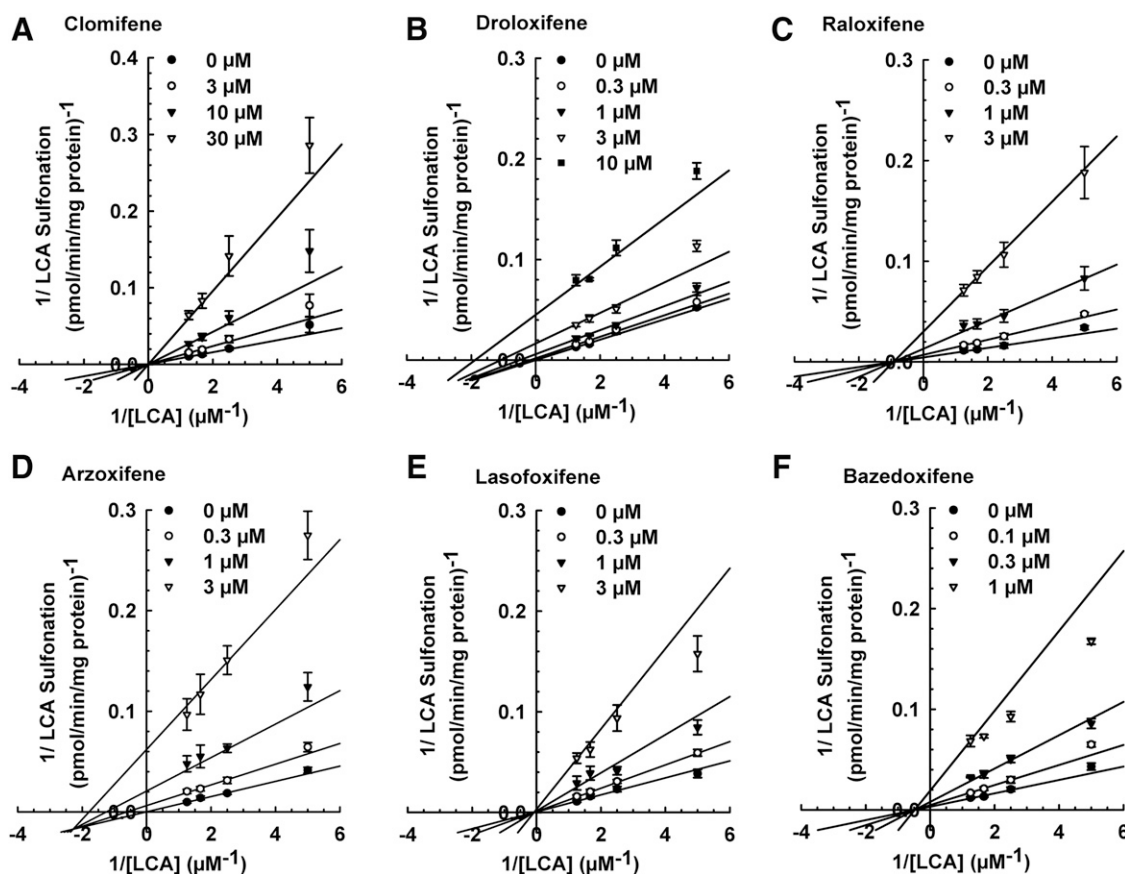
$IC_{50}$  values and minimum inhibitory concentration (MIC) for the inhibition of human liver cytosolic lithocholic acid (LCA) sulfonation by selective estrogen receptor modulator (SERMs)

Data are expressed as mean  $\pm$  S.E.M. of three to four independent experiments conducted in duplicate.

Chemical Class	Chemical	$IC_{50}$ ( $\mu$ M) LCA Sulfonation	$IC_{50}$ ( $\mu$ M) GLCA Sulfonation	$IC_{50}$ ( $\mu$ M) TLCA Sulfonation	MIC ( $\mu$ M) LCA Sulfonation	MIC ( $\mu$ M) GLCA Sulfonation	MIC ( $\mu$ M) TLCA Sulfonation
Triphenylethylene	Clomifene	$3.2 \pm 0.5$	$3.6 \pm 0.3$	$1.5 \pm 0.2$	3	1	0.3
Triphenylethylene	Tamoxifen	$16.3 \pm 5.3$	$13.7 \pm 1.6$	$10.1 \pm 0.8$	10	30	30
Triphenylethylene	Toremifene	$9.2 \pm 5.3$	$11.0 \pm 1.2$	$3.2 \pm 1.1$	10	3	3
Triphenylethylene	Ospemifene	$3.0 \pm 0.2$	$2.55 \pm 0.02$	$2.3 \pm 0.4$	10	3	<0.1
Triphenylethylene	Droloxifene	$1.5 \pm 0.2$	$1.5 \pm 0.1$	$0.75 \pm 0.01$	1	1	0.1
Benzothiophene	Raloxifene	$0.28 \pm 0.04$	$0.41 \pm 0.03$	$0.18 \pm 0.02$	0.1	0.3	0.1
Benzothiophene	Arzoxifene	$0.29 \pm 0.04$	$0.20 \pm 0.01$	$0.08 \pm 0.01$	0.3	0.1	0.01
Tetrahydronaphthale	Lasofoxifene	$1.0 \pm 0.1$	$0.50 \pm 0.01$	$0.25 \pm 0.01$	0.3	0.1	<0.01
Tetrahydronaphthale	Nafoxidine	$12.2 \pm 2.1^a$	$6.6 \pm 5.2^a$	$1.4 \pm 0.7^a$	100	>100	10
Indole	Bazedoxifene	$0.21 \pm 0.03$	$0.29 \pm 0.01$	$0.13 \pm 0.02$	0.03	0.1	0.3
Benzopyran	Acolbifene	$12.9 \pm 3.2$	$10.1 \pm 0.7$	$7.0 \pm 0.3$	10	10	3

GLCA, glycolithocholic acid; TLCA, tauroolithocholic acid.

<sup>a</sup>Complete inhibition was not reached (Figs. 5–7).



**Fig. 8.** Lineweaver-Burk plots for the inhibition of human liver cytosolic LCA sulfonation by SERMs. Human liver cytosol (80  $\mu\text{g}$  of protein) was incubated with LCA (0.2, 0.4, 0.6, or 0.8  $\mu\text{M}$ ) and with varying concentrations of a SERM [clomifene (0, 3, 10, or 30  $\mu\text{M}$ ) (A), droloxifene (0, 0.3, 1, 3, 10, or 30  $\mu\text{M}$ ) (B), raloxifene (0, 0.3, 1, or 3  $\mu\text{M}$ ) (C), arzoxifene (0, 0.3, 1, or 3  $\mu\text{M}$ ) (D), lasofoxifene (0, 0.3, 1, or 3  $\mu\text{M}$ ) (E), or bazedoxifene (0, 0.1, 0.3, or 1  $\mu\text{M}$ ) (F)] at 37°C for 15 minutes. Data are expressed as mean  $\pm$  S.E.M. for three to four independent experiments conducted in duplicate.

lasofoxifene ( $0.8 \pm 0.1 \mu\text{M}$ ), and arzoxifene ( $0.8 \pm 0.2 \mu\text{M}$ ) were an order of magnitude less than those for droloxifene ( $4.6 \pm 0.7 \mu\text{M}$ ) and clomifene ( $5.8 \pm 0.5 \mu\text{M}$ ).

**Comparative Inhibition of Human Liver Cytosolic LCA Sulfonation by Toremifene and Its Oxidative Metabolites.** The inhibitory effects of toremifene and its oxidative metabolites (*N*-desmethyl-4-hydroxytoremifene, *N*-desmethyltoremifene, 4-hydroxytoremifene, or 4'-hydroxytoremifene) (Fig. 1) on human liver cytosolic LCA sulfonation were compared. At 5  $\mu\text{M}$ , 4'-hydroxytoremifene inhibited the reaction to a greater extent than the parent drug, toremifene (Fig. 9A). At 10  $\mu\text{M}$ , the order of inhibition of LCA sulfonation was *N*-desmethyl-4-hydroxytoremifene > 4-hydroxytoremifene > toremifene > *N*-desmethyltoremifene (Fig. 9A). As shown in the concentration-response curves (Fig. 9, B–F) and the calculated  $IC_{50}$  values (Table 4), 4'-hydroxytoremifene and

4-hydroxytoremifene were somewhat more potent inhibitors of LCA sulfonation than the non-hydroxy-substituted toremifene and *N*-desmethyltoremifene, as well as *N*-desmethyl-4-hydroxytoremifene. Similar MIC values were obtained for 4-hydroxytoremifene and 4'-hydroxytoremifene in the inhibition of human liver cytosolic LCA sulfonation (Table 4).

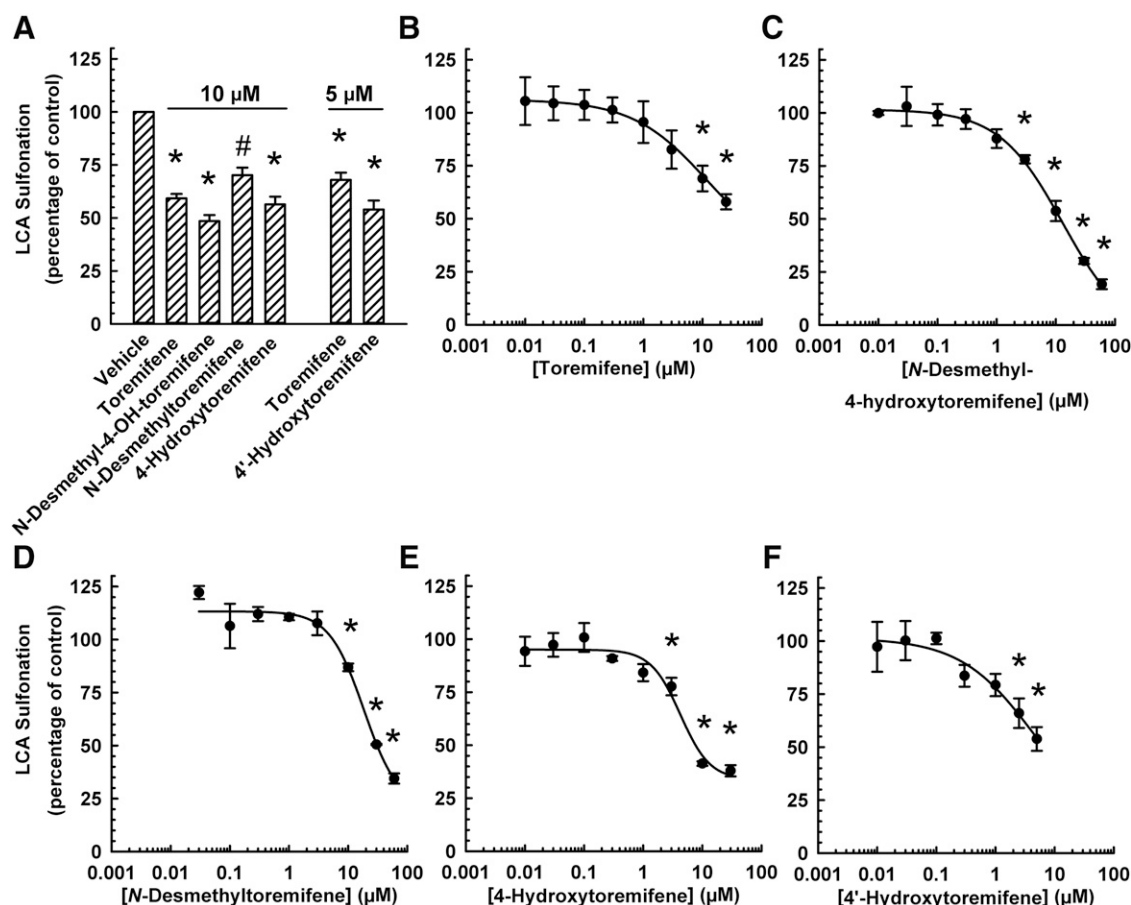
**Comparative Inhibition of Human Liver Cytosolic LCA Sulfonation by Lasofoxifene and Its Structural Analogs.** We compared the inhibitory effects of lasofoxifene and its analogs, namely, 7-methoxylasofoxifene and *cis*-4-(1,2,3,4-tetrahydro-6-methoxy-2-phenyl-1-naphthalenyl)-phenol (Fig. 1), on human liver cytosolic LCA sulfonation. As shown in Fig. 10A, at a concentration of 10  $\mu\text{M}$ , both analogs inhibited LCA sulfonation to a lesser extent than lasofoxifene. Concentration-response experiments showed that lasofoxifene (Fig. 10B) was more efficacious than 7-methoxylasofoxifene

TABLE 3

Apparent  $K_i$  values and mode of inhibition of human liver cytosolic lithocholic acid (LCA) sulfonation by selective estrogen receptor modulators (SERMs)

Data are expressed as mean  $\pm$  S.E.M. of three to four independent experiments conducted in duplicate.

Chemical Class	Chemical	Apparent $K_i$ ( $\mu\text{M}$ )	Mode of Inhibition	Ratio of Apparent $K_i$ to Apparent $K_m$
Indole	Bazedoxifene	$0.2 \pm 0.03$	Noncompetitive (full)	0.18
Benzothiophene	Raloxifene	$0.5 \pm 0.04$	Noncompetitive (full)	0.45
Tetrahydronaphthalene	Lasofoxifene	$0.8 \pm 0.1$	Noncompetitive (full)	0.73
Benzothiophene	Arzoxifene	$0.8 \pm 0.2$	Mixed (full)	0.73
Triphenylethylene	Droloxifene	$4.6 \pm 0.7$	Mixed (partial)	4.2
Triphenylethylene	Clomifene	$5.8 \pm 0.5$	Competitive (full)	5.3



**Fig. 9.** Comparative inhibitory effect of toremifene metabolites on human liver cytosolic LCA sulfonation. (A) Human liver cytosol (80  $\mu\text{g}$  protein) was incubated with LCA (0.75  $\mu\text{M}$ ; 0.25% v/v methanol) and toremifene (10  $\mu\text{M}$ ), its metabolites (each 10  $\mu\text{M}$ ), toremifene (5  $\mu\text{M}$ ) or 4'-hydroxy toremifene (5  $\mu\text{M}$ ), or vehicle (0.25% v/v methanol) at 37°C for 15 minutes. (B–F) Human liver cytosol (80  $\mu\text{g}$  of protein) was incubated with LCA (0.75  $\mu\text{M}$ ; 0.25% v/v methanol) and with varying concentrations of toremifene (0.01–25  $\mu\text{M}$ ) (B), *N*-desmethyl-4-hydroxytoremifene (0.01–60  $\mu\text{M}$ ) (C), *N*-desmethyltoremifene (0.01–60  $\mu\text{M}$ ) (D), 4-hydroxytoremifene (0.01–30  $\mu\text{M}$ ) (E), 4'-hydroxytoremifene (0.01–5  $\mu\text{M}$ ) (F), or vehicle (0.25% v/v methanol) at 37°C for 15 minutes. Data are expressed as percentage of LCA sulfonation in the vehicle-treated control group and expressed as mean  $\pm$  S.E.M. for three independent experiments conducted in duplicate. \*Significantly different from the vehicle-treated control group ( $P < 0.05$ ). #Significantly different from the vehicle-treated control group and the toremifene-treated group ( $P < 0.05$ ).

(Fig. 10C) or *cis*-4-(1,2,3,4-tetrahydro-6-methoxy-2-phenyl)-naphthalene)phenol (Fig. 10D) in the inhibition of human liver cytosolic LCA sulfonation. 7-Methoxylasofloxifene was

approximately 3 times less potent than lasofloxifene, whereas *cis*-4-(1,2,3,4-tetrahydro-6-methoxy-2-phenyl)naphthalene)phenol had similar inhibitory potency to that of lasofloxifene, as

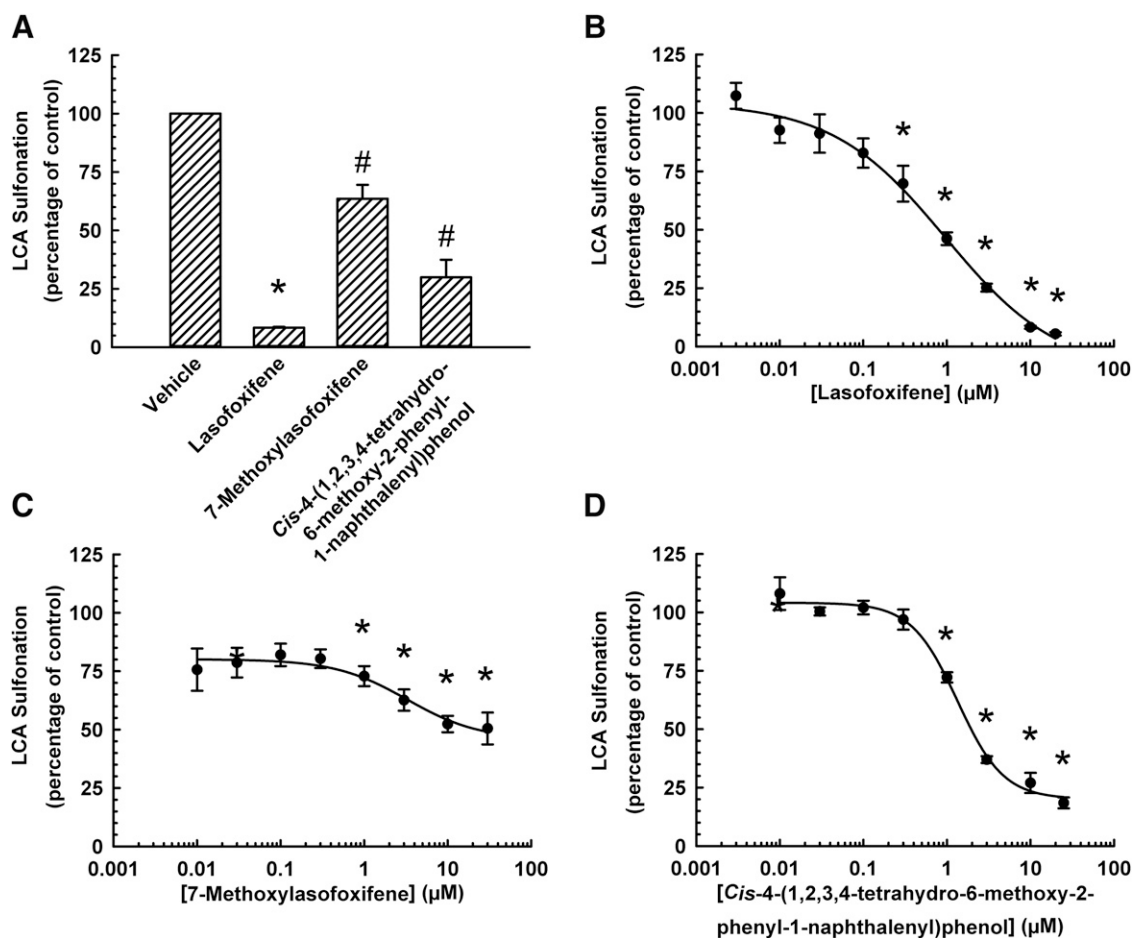
TABLE 4

IC<sub>50</sub> values and minimum inhibitory concentration (MIC) for the inhibition of human liver cytosolic (LCA) sulfonation by SERMs

Data are expressed as mean  $\pm$  S.E.M. of three independent experiments conducted in duplicate.

Chemical	IC <sub>50</sub> ( $\mu\text{M}$ )	MIC ( $\mu\text{M}$ )
Toremifene and metabolites/analogs		
4'-Hydroxytoremifene	1.8 $\pm$ 1.0	2.5
4-Hydroxytoremifene	4.4 $\pm$ 0.3	3
Toremifene	9.2 $\pm$ 5.3	10
<i>N</i> -Desmethyl-4-hydroxytoremifene	12.9 $\pm$ 1.2	3
<i>N</i> -Desmethyltoremifene	18.4 $\pm$ 1.2	10
Lasofloxifene and analogs		
Lasofloxifene	1.0 $\pm$ 0.1	0.3
<i>Cis</i> -4-(1,2,3,4-tetrahydro-6-methoxy-2-phenyl-1-naphthalenyl)phenol	1.4 $\pm$ 0.1	1
7-Methoxylasofloxifene	2.9 $\pm$ 1.0	1
Bazedoxifene and metabolites/analogs		
Bazedoxifene <i>N</i> -oxide	0.06 $\pm$ 0.01*	0.003
Des(1-azepanyl)ethylbazedoxifene	0.16 $\pm$ 0.02	0.1
Bazedoxifene	0.21 $\pm$ 0.03	0.03

\*Significantly different from the parent drug-treated group ( $P < 0.05$ ).



**Fig. 10.** Comparative inhibitory effect of lasofoxifene derivatives on human liver cytosolic LCA sulfonation. (A) Human liver cytosol (80  $\mu$ g of protein) was incubated with LCA (0.75  $\mu$ M; 0.25% v/v methanol) and lasofoxifene or its derivatives (each at 10  $\mu$ M) or vehicle (0.25% v/v methanol) at 37°C for 15 minutes. (B–D) Human liver cytosol (80  $\mu$ g of protein) was incubated with LCA (0.75  $\mu$ M; 0.25% v/v methanol) and with varying concentrations of lasofoxifene (0.01–20  $\mu$ M) (B), 7-methoxy lasofoxifene (0.01–30  $\mu$ M) (C), *cis*-4-(1,2,3,4-tetrahydro-6-methoxy-2-phenyl-1-naphthalenyl)phenol (0.01–25  $\mu$ M) (D), or vehicle (0.25% v/v methanol) at 37°C for 15 minutes. Data are expressed as percentage of LCA sulfonation in the vehicle-treated control group and expressed as mean  $\pm$  S.E.M. for three independent experiments conducted in duplicate. \*Significantly different from the vehicle-treated control group ( $P < 0.05$ ). #Significantly different from the vehicle-treated control group and the lasofoxifene-treated group ( $P < 0.05$ ).

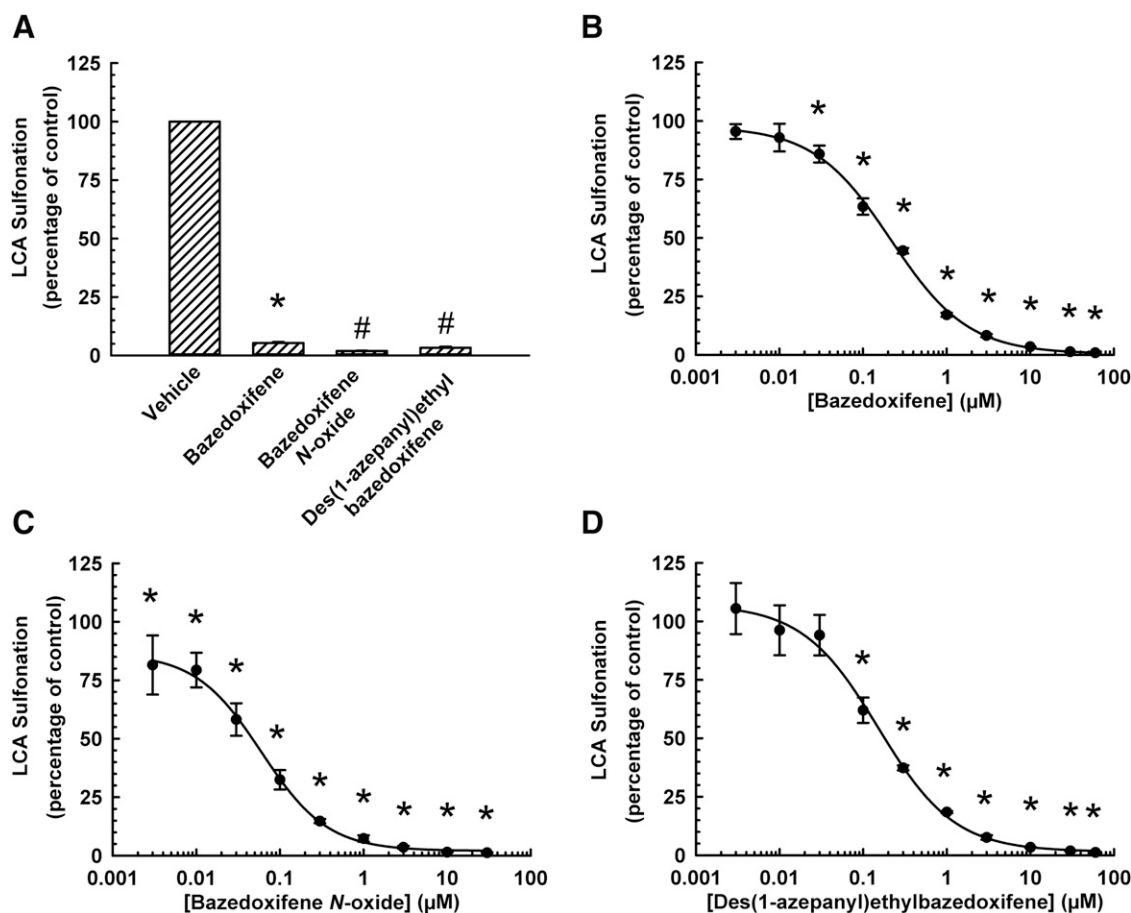
indicated by the  $IC_{50}$  values (Table 4). The two analogs of lasofoxifene also had a greater MIC than lasofoxifene (Table 4).

**Comparative Inhibition of Human Liver Cytosolic LCA Sulfonation by Bazedoxifene and Its Metabolites or Structural Analogs.** The inhibitory effects of bazedoxifene and its bazedoxifene *N*-oxide and des(1-azepanyl)-ethylbazedoxifene metabolites (Fig. 1) on human liver cytosolic LCA sulfonation were compared. As shown in Fig. 11A, at a concentration of 10  $\mu$ M, both bazedoxifene *N*-oxide and des(1-azepanyl)ethylbazedoxifene inhibited LCA sulfonation to a greater extent than the parent drug, bazedoxifene. Concentration-response experiments (Fig. 11, B–D) and  $IC_{50}$  values (Table 4) indicated that bazedoxifene *N*-oxide was somewhat more potent than the parent drug, bazedoxifene, whereas des(1-azepanyl)-ethylbazedoxifene inhibited LCA sulfonation with comparable potency as the parent drug. Bazedoxifene *N*-oxide had a lesser minimum MIC than bazedoxifene, which in turn had a lesser MIC than des(1-azepanyl)ethylbazedoxifene (Table 4).

**Effect of Preincubation of Human Liver Cytosol with a SERM on the Inhibition of LCA Sulfonation by a SERM.** To investigate whether SERMs are time-dependent inactivators of SULT2A1-catalyzed LCA sulfonation, clomifene, tamoxifen, toremifene, ospemifene, droloxifene, raloxifene,

arzofoxifene, lasofoxifene, nafoxidine, bazedoxifene, or acolbifene was preincubated with human liver cytosol and PAPS at 37°C for 0, 30, 60, or 90 minutes. Subsequently, an aliquot of primary incubation mixture was transferred into a secondary incubation mixture containing LCA and PAPS. As shown in Supplemental Fig. S3, preincubation of a SERM with human liver cytosol did not further increase the inhibitory effect of that SERM on LCA sulfonation.

**In Situ Effect of SERMs on LCA Sulfonation in Cultured HepG2 Human Hepatocellular Carcinoma Cells.** To determine whether SERMs inhibit LCA sulfonation in a whole-cell system, HepG2 cells were used as a cellular model because they express SULT2A1 (Westerink and Schoonen, 2007; Kurogi et al., 2012). Our initial experiment showed increasing formation of LCA-S metabolite in LCA-treated HepG2 cells as a function of incubation time, indicating the abundance and functionality of SULT2A1 in HepG2 cells. The rate of reaction in the LCA-treated control group was  $3.19 \pm 0.41$  pmol/min per milligram of total cellular protein (Fig. 12A),  $0.91 \pm 0.05$  pmol/min per milligram of total cellular protein (Fig. 12B), and  $3.36 \pm 0.24$  pmol/min per milligram of total cellular protein (Fig. 12C). When the cells were treated with LCA substrate and a SERM for 15 minutes,



**Fig. 11.** Comparative inhibitory effect of bazedoxifene metabolite/analogs on human liver cytosolic LCA sulfonation. (A) Human liver cytosol (80  $\mu$ g of protein) was incubated with LCA (0.75  $\mu$ M; 0.25% v/v methanol) and bazedoxifene or its metabolite/analogs (each at 10  $\mu$ M) or vehicle (0.25% v/v methanol) at 37°C for 15 minutes. (B–D) Human liver cytosol (80  $\mu$ g of protein) was incubated with LCA (0.75  $\mu$ M; 0.25% v/v methanol) and with varying concentrations of bazedoxifene (0.01–60  $\mu$ M) (B), bazedoxifene N-oxide (0.003–30  $\mu$ M) (C), des(1-azepanyl)ethyl bazedoxifene (0.003–60  $\mu$ M) (D), or vehicle (0.25% v/v methanol) at 37°C for 15 minutes. Data are expressed as mean  $\pm$  S.E.M. for three independent experiments conducted in duplicate. \*Significantly different from the vehicle-treated control group ( $P < 0.05$ ). #Significantly different from the vehicle-treated control group and the bazedoxifene-treated group ( $P < 0.05$ ).

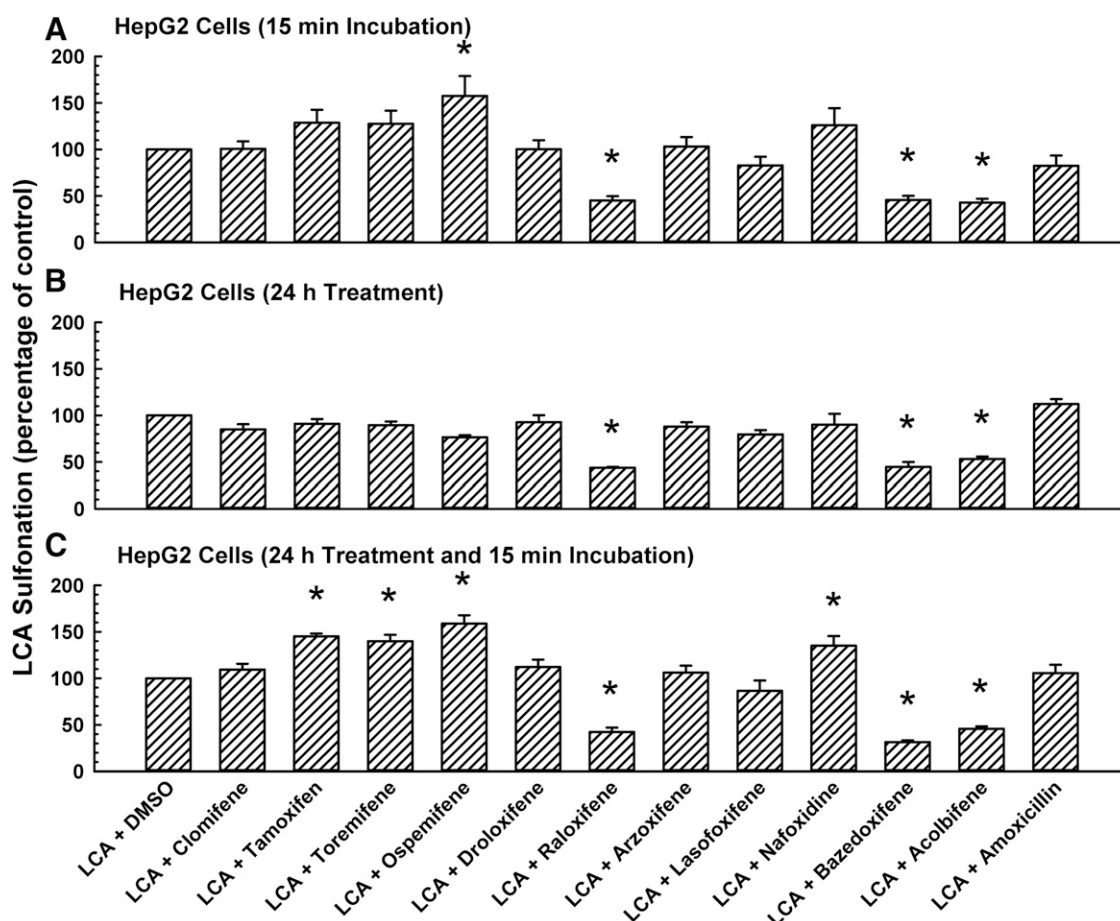
raloxifene, bazedoxifene, and acolbifene, but not the other SERMs, decreased the extent of LCA sulfonation (Fig. 12A).

To determine whether the inhibitory effect of raloxifene, bazedoxifene, and acolbifene on LCA-S formation persisted beyond a 15-minute incubation period (Fig. 12A), HepG2 cells were cotreated with LCA and a SERM for 24 hours. As shown in Fig. 12B, the LCA-S level was decreased in the presence of raloxifene, bazedoxifene, or acolbifene, but not by the other SERMs. To further corroborate these findings, LCA- and SERM-cotreated cells were washed and incubated in situ with another dose of LCA and a SERM for 15 minutes. As shown in Fig. 12C, raloxifene, bazedoxifene, and acolbifene, but none of the other SERMs, decreased LCA-S formation. Amoxicillin, which does not affect LCA sulfonation in enzymatic incubations containing human liver cytosol (Bamforth et al., 1992), had no effect on LCA sulfonation in cultured HepG2 cells, regardless of the experimental condition (Fig. 12).

## Discussion

Our study represents the first detailed investigation demonstrating that the triphenylethylene, benzothiophene, tetrahydronaphthalene, indole, and benzopyran classes of SERMs

(Fig. 1) inhibited the sulfonation of DHEA (a prototypic substrate of SULT2A1), LCA, GLCA, and TLCA in enzymatic incubations containing human liver cytosol or recombinant SULT2A1. In general, the benzothiophene, tetrahydronaphthalene, and indole classes of SERMs were more potent than the triphenylethylene and benzopyran classes of SERM in the inhibition of human liver cytosolic LCA, GLCA, and TLCA sulfonation. The individual SERMs inhibited the sulfonation of LCA and GLCA with somewhat similar potency and efficacy, but they were slightly more potent (lower  $IC_{50}$  values) in inhibiting the sulfonation of TLCA than those of LCA and GLCA. The amidation of LCA, however, did not greatly decrease the susceptibility of LCA to inhibition by individual SERMs. Among the SERMs investigated, bazedoxifene, raloxifene, lasofoxifene, and arzoxifene were identified as the most potent and efficient inhibitors of human liver cytosolic LCA sulfonation, as judged by the apparent  $K_i$  values and ratio of apparent  $K_i$  to apparent  $K_m$ , respectively. Bazedoxifene, with a five-member pyrrole, and raloxifene, with a five-member thiophene, were stronger inhibitors than acolbifene, which is a benzopyran and has a fused six-membered pyran ring (Fig. 1), of LCA, GLCA, and TLCA sulfonation, suggesting the importance of a five-membered pyrrole and thiophene in inhibiting



**Fig. 12.** In situ effect of SERMs on LCA sulfonation in cultured HepG2 cells. (A) Cultured cells were incubated with LCA ( $0.5 \mu\text{M}$ ) and a SERM (clomifene, tamoxifen, toremifene, ospemifene, droloxifene, raloxifene, arzoxifene, lasofoxifene, nafoxidine, bazedoxifene, or acolbifene;  $10 \mu\text{M}$  each), LCA and amoxicillin ( $1 \text{ mM}$ ; negative control), LCA and vehicle, or vehicle ( $0.1\% \text{ v/v}$  DMSO) at  $37^\circ\text{C}$  for 15 minutes. The incubation mixture and cell lysates were harvested. (B) Cultured cells were cotreated with LCA ( $0.5 \mu\text{M}$ ) and a SERM (as listed above,  $10 \mu\text{M}$  each), LCA and amoxicillin ( $1 \text{ mM}$ ; negative control), LCA and vehicle, or vehicle ( $0.1\% \text{ v/v}$  DMSO) at  $37^\circ\text{C}$  for 24 hours. The culture medium was harvested. (C) At the end of the 24-hour treatment period with LCA and a SERM, cultured cells were incubated with LCA ( $0.5 \mu\text{M}$ ) and a SERM (as listed above,  $10 \mu\text{M}$  each), LCA and amoxicillin ( $1 \text{ mM}$ ; negative control), LCA and vehicle, or vehicle ( $0.1\% \text{ v/v}$  DMSO) at  $37^\circ\text{C}$  for 15 minutes. The incubation mixture and cell lysates were harvested. (A–C) The total metabolite level was quantified and normalized to the total cellular protein in each well, as determined by a total protein assay. Data are expressed as percentage of LCA sulfonation in the LCA-treated control group and expressed as mean  $\pm$  S.E.M. for four or five independent experiments conducted in duplicate. \*Significantly different from the LCA-treated control group ( $P < 0.05$ ). The rate of reaction in the LCA-treated control group was  $3.19 \pm 0.41 \text{ pmol/min per milligram}$  of total cellular protein (A),  $0.91 \pm 0.05 \text{ pmol/min per milligram}$  of total cellular protein (B), and  $3.36 \pm 0.24 \text{ pmol/min per milligram}$  of total cellular protein (C).

LCA, GLA, or TLCA sulfonation by bazedoxifene and raloxifene, respectively.

Among the triphenylethylene class of SERMs, droloxifene, a hydroxylated derivative of tamoxifen, was the most potent inhibitor of human liver cytosolic LCA, GLCA, and TLCA sulfonation, with approximately 10 times greater potency than that of tamoxifen. A chlorine-substituted triphenylethylene derivative, clomifene, which does not have a hydroxyl group, inhibited LCA, GLCA, and TLCA sulfonation, suggesting that the chlorine group may form a bond with a carbonyl oxygen of amino acid residues, as shown previously that halogens can interact with a carbonyl oxygen (Bissantz et al., 2010). In contrast, toremifene, which contains a chloroethyl group instead of a chloro group on the triphenylethylene scaffold, was a weaker inhibitor compared with droloxifene and clomifene. Ospemifene, where the dimethylamino group of toremifene is replaced with a polar hydroxyl group while retaining the chloroethyl substitution of toremifene, inhibited the sulfonation of LCA, GLA, or TLCA with

greater inhibitory potency than toremifene. This result may be due to the interaction between the polar hydroxyl group and the amino acid residues of SULT2A1. As assessed using toremifene (triphenylethylene class) and its metabolites, the addition of a hydroxyl group on the triphenylethylene moiety and *N*-demethylation increases the extent of inhibition of LCA sulfonation. Furthermore, 4'-hydroxytoremifene and 4-hydroxytoremifene inhibited LCA sulfonation to a similar extent, indicating that the substitution of hydroxyl group on either of the benzene rings in the triphenylethylene moiety does not affect SULT2A1 catalytic activity. Consistent with our finding, a previous study reported that endoxifen, an *N*-desmethyl-4-hydroxy derivative of tamoxifen, is more potent than tamoxifen as an inhibitor of SULT2A1 catalytic activity (Squirewell et al., 2014).

Methylation of the 7-hydroxyl group of lasofoxifene attenuated the inhibitory activity of lasofoxifene on human liver cytosolic LCA sulfonation, thereby indicating the importance of the hydroxyl group in the inhibition of LCA sulfonation by

lasofoxifene. Another tetrahydronaphthalene derivative, nafoxidine, which has a 7-methoxy group and a double bond between the two phenyl rings, showed weak or negligible inhibition of LCA, GLCA, or TLCA sulfonation. The finding suggests that methylation of a hydroxyl group and a double bond between two phenyl rings are not favorable for the inhibition. A plausible explanation is that the double bond in nafoxidine may constrain the free movement of phenyl groups, which results in loss of hydrophobic interaction of nafoxidine with the amino acid residues of SULT2A1. In contrast, *cis*-4-(1,2,3,4-tetrahydro-6-methyl-2-phenyl)naphthalene)phenol, which also has a 7-methoxy group but without a *O*-ethylpyrrolidine, displayed similar inhibitory potency as that of lasofoxifene, suggesting that *O*-ethylpyrrolidine group is not crucial for the inhibition of LCA sulfonation by lasofoxifene and the phenolic hydroxyl group in the phenyl ring at the 1-tetrahydronaphthalene position helps to prevent the loss of inhibitory activity caused by the loss of the 7-hydroxy group. Bazedoxifene and its metabolite or analog contain hydroxy groups in the indole ring and the aromatic rings attached to the indole ring. Compared with bazedoxifene, bazedoxifene *N*-oxide was approximately 3 times more potent, whereas des(1-azepanyl)ethylbazedoxifene was equipotent, suggesting that 1-(azepanyl)ethyl moiety is not crucial for the inhibition of LCA sulfonation by bazedoxifene. Taken together, the findings from the structural analogs of lasofoxifene and bazedoxifene indicate that the hydroxyl groups on the chemical scaffold are important for the inhibition of human SULT2A1-catalyzed LCA sulfonation.

Select SERMs inhibited human liver cytosolic LCA sulfonation by various modes of direct inhibition involving the parent drug, as exemplified by the finding that bazedoxifene, raloxifene, and lasofoxifene inhibited LCA sulfonation by a noncompetitive mode, whereas droloxifene and arzoxifene inhibited it by mixed mode and clomifene by competitive mode. Our study also showed that these SERMs do not inhibit SULT2A1-catalyzed human liver cytosolic LCA sulfonation by mechanism-based inactivation involving a reactive intermediate. Previous studies reported the inactivation of a rat sulfotransferase enzyme by chemicals such as *N*-hydroxy-2-acetylaminofluorene (Mangold et al., 1990; Ringer et al., 1992). The inactivation of sulfotransferase occurs as a result of the formation of reactive metabolites (formed when the sulfate group is cleaved off and resulting in an electrophilic cation). This cleavage of sulfate group is facilitated if the resulting cation is stabilized by resonance, as shown by sulfuric acid esters of allylic or benzylic alcohols (Glatt, 1997, 2000). A plausible explanation for the lack of mechanism-based inactivation by SERMs is the absence of inductive effect or resonance in the phenolic hydroxyl group to stabilize the reactive metabolite. Therefore, reactive electrophilic cations generated by SERMs may not exist to form covalent bonds with proteins.

Among the SERMs investigated, raloxifene, bazedoxifene, and acolbifene are efficacious inhibitors *in situ* in a cultured cell model. Raloxifene and bazedoxifene are efficacious inhibitors of LCA sulfonation in cell-free and cell culture models, whereas clomifene, tamoxifene, toremifene, ospemifene, droloxifene, arzoxifene, lasofoxifene, and nafoxidine inhibited only LCA sulfonation in a cell-free model. This result could be because some of the SERMs may be metabolized to other metabolites that do not inhibit LCA sulfonation. In contrast,

acolbifene is a weak inhibitor in human liver cytosol and recombinant enzyme but showed stronger inhibition in cultured cells. A plausible explanation could be that acolbifene may be metabolized to a metabolite capable of inhibiting the catalytic activity of SULT2A1. None of the SERMs investigated in the present study increased the extent of LCA-S formation to any appreciable extent, suggesting that these SERMs are not inducers of SULT2A1 under the experimental conditions used (Fig. 12).

The potential *in vivo* clinical relevance of the *in vitro* inhibition of SULT2A1-catalyzed sulfonation by SERMs depends on various factors, including the magnitude of the *in vitro* apparent  $K_i$  and the *in vivo* inhibitor concentration achieved at the site of a potential interaction, such as the liver and small intestine, which express SULT2A1 (Riches et al., 2009); however, hepatic concentrations of SERMs in humans are not known. For an orally administered drug, the intraluminal drug concentration in the gastrointestinal tract can be estimated by dividing the dose with an intestinal volume of 250 ml (Food and Drug Administration, 2017, <https://www.fda.gov/downloads/Drugs/Guidances/UCM581965.pdf>). The intraluminal concentration of an orally administered SERM is estimated to reach approximately 170  $\mu\text{M}$  for 20 mg of bazedoxifene and 500  $\mu\text{M}$  for 60 mg of raloxifene. These concentrations are considerably greater than the apparent  $K_i$  values of bazedoxifene (0.2  $\mu\text{M}$ ) and raloxifene (0.5  $\mu\text{M}$ ) we obtained in the present study. Overall, the present study provides a basis for future human studies investigating the effect of an orally administered SERM on the clearance of a SULT2A1 substrate.

In conclusion, among the SERMs investigated, bazedoxifene, raloxifene, lasofoxifene, and arzoxifene were the most potent *in vitro* inhibitors of human liver cytosolic LCA, GLCA, and TLCA sulfonation, which were catalyzed by SULT2A1 but not by SULT2B1b or SULT1E1. Bazedoxifene, raloxifene, and acolbifene inhibited LCA sulfonation in cultured HepG2 cells. The metabolites or structural analogs of toremifene, lasofoxifene, and bazedoxifene revealed the structural features that contribute to the inhibition of LCA sulfonation, thereby providing insight into strategies that may help in the design of new SERMs to avoid inhibiting the sulfonation of SULT2A1 substrates such as LCA, GLCA, and TLCA, which may lead to adverse effects. Previously, drug-induced intrahepatic cholestasis has been associated with inhibition of bile acid biotransformation (Sharanek et al., 2017). Therefore, the present findings provides a biochemical basis for the onset of cholestasis reported in patients on specific SERM therapy such as tamoxifen (Mazokopakis et al., 2007) and raloxifene (Vilches et al., 1998). Concentrations of bile acids may be increased by bile acid malabsorption (Nolan et al., 2013), high-fat diet (Bernstein et al., 2005), hepatic diseases or failure (Ceryak et al., 1998), and chronic and relapsing inflammatory bowel diseases (e.g., Crohn disease and ulcerative colitis) (Rogler, 2012). Therefore, inhibition of LCA clearance by a SERM may further increase bile acid concentrations under these conditions.

#### Authorship Contributions

Participated in research design: Bansal, Lau.

Conducted experiments: Bansal, Lau.

Performed data analysis: Bansal, Lau.

Wrote or contributed to the writing of the manuscript: Bansal, Lau.

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**Inhibition of Human SULT2A1-Catalyzed Sulfonation of Lithocholic Acid, Glycolithocholic Acid, and Tauroolithocholic Acid by Selective Estrogen Receptor Modulators and Various Analogues and Metabolites**

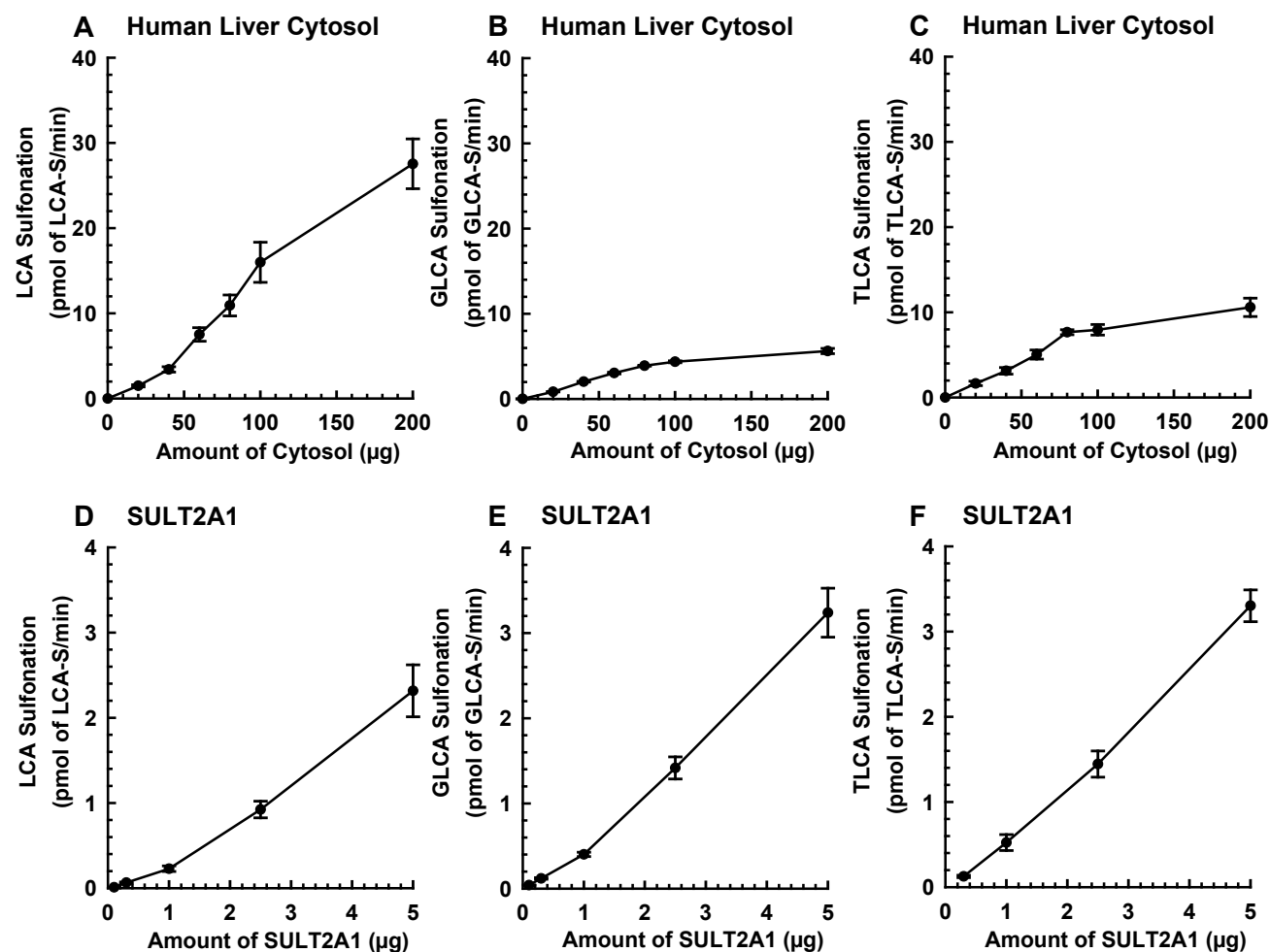
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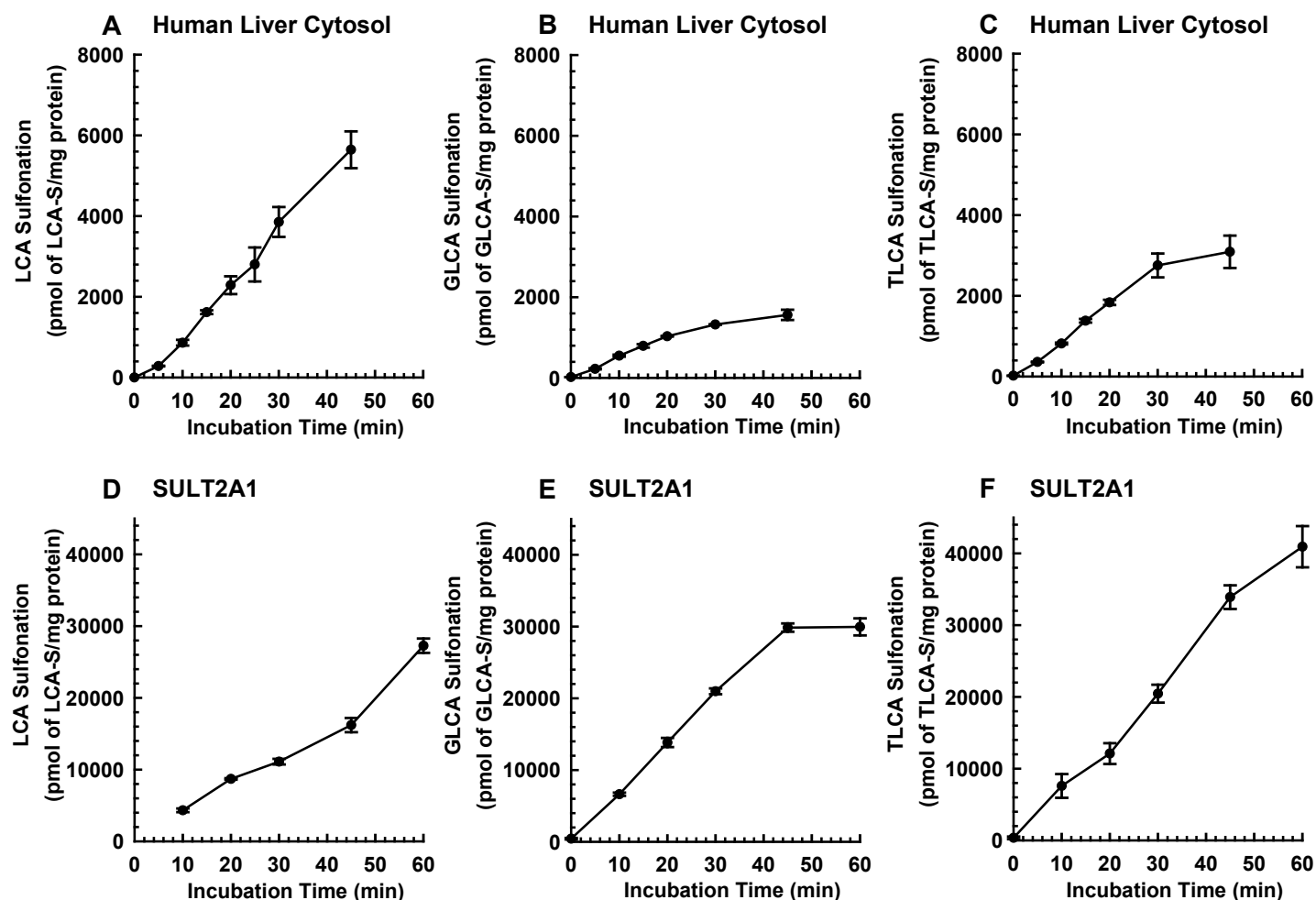
## SUPPLEMENTAL TABLE S1

Optimized mass spectrometric parameters for the analysis of LCA-S, GLCA-S, GLCA, TLCA-S, TLCA, and cholic acid (internal standard) by UPLC-MS/MS.

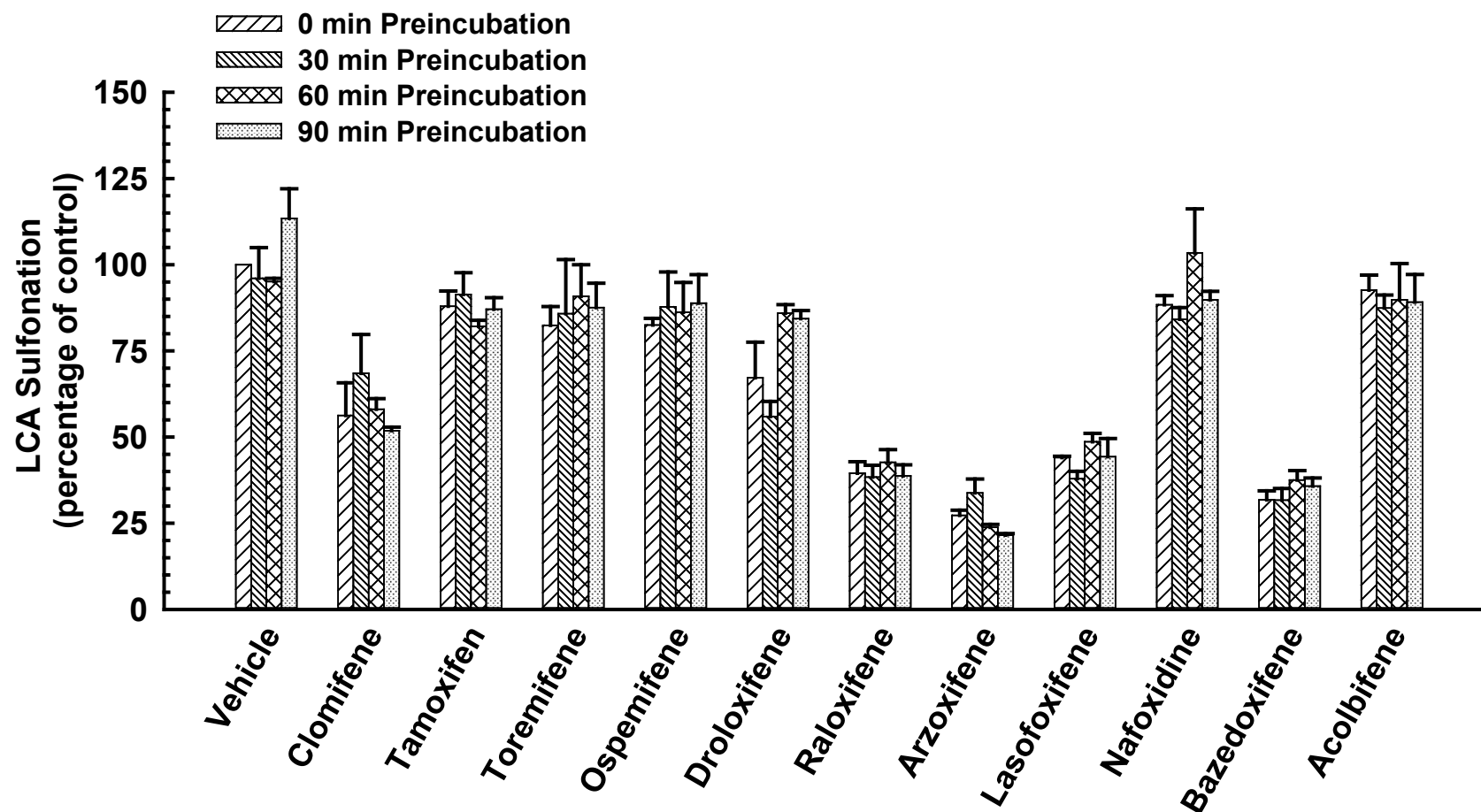
Chemical	<i>m/z</i> transition	Declustering potential	Entrance potential	Collision energy	Collision cell exit potential	Dwell time	Curtain gas	Collision- activated dissociation gas	Ion spray voltage	Ion source temperature	Ion source gas 1	Ion source gas 2
	<i>m/z</i>	<i>V</i>	<i>V</i>	<i>V</i>	<i>V</i>	<i>ms</i>	<i>psi</i>	<i>psi</i>	<i>V</i>	<i>°C</i>	<i>psi</i>	<i>psi</i>
LCA-S	455.3 → 97.0	-160	-9.6	-113	-5	300	20	6	-4500	550	50	50
GLCA-S	512.4 → 432.1	-145	-10	-45	-10	200	20	6	-4500	600	50	50
GLCA	432.3 → 74.0	-160	-10	-75	-10	200	20	6	-4500	600	50	50
TLCA-S	280.7 → 97.0	-110	-5	-37	-7	200	20	6	-4500	600	50	50
TLCA	482.3 → 80.0	-230	-7	-120	-5	200	20	6	-4500	600	50	50
Cholic acid	407.3 → 343.3	-170	-9.8	-44	-5.9	200	20	6	-4500	550 (LCA) or 600 (GLCA/TLCA)	50	50



**Supplemental Fig. S1.** Relationship between amount of human liver cytosolic protein or recombinant SULT2A1 protein and LCA, GLCA, or TLCA sulfonation. Varying amount of human liver cytosol (0, 20, 40, 60, 80, 100, or 200 μg protein) (A-C) or SULT2A1 (0, 0.1, 0.3, 1, 2.5, or 5 μg protein) (D-F) was incubated with LCA (1 μM; 0.5% v/v methanol) (A), GLCA (0.75 μM; 0.5% v/v methanol) (B), TLCA (0.75 μM; 0.5% v/v methanol) (C), LCA (2.5 μM; 0.5% v/v methanol) (D), GLCA (1 μM; 0.5% v/v methanol) (E), or TLCA (1 μM; 0.5% v/v methanol) (F) at 37°C for 10 min (A), 15 min (B-C), or 30 min (D-F). Data are expressed as mean ± S.E.M. of three independent experiments conducted in duplicate.



**Supplemental Fig. S2.** Relationship between incubation time and LCA, GLCA, or TLCA sulfonation catalyzed by human liver cytosol or recombinant SULT2A1. Human liver cytosol (60 µg protein) (A-B), human liver cytosol (40 µg protein) (C), SULT2A1 (5 µg protein) (D), or SULT2A1 (2.5 µg protein) (E-F) was incubated with LCA (1 µM; 0.5% v/v methanol) (A), GLCA (0.75 µM; 0.5% v/v methanol) (B), TLCA (0.75 µM; 0.5% v/v methanol) (C), LCA (2.5 µM; 0.5% v/v methanol) (D), GLCA (1 µM; 0.5% v/v methanol) (E), or TLCA (1 µM; 0.5% v/v methanol) (F) at 37°C for 0, 5, 10, 15, 20, 25, 30, or 45 min (A-C) or 0, 10, 20, 30, 45, or 60 min (D-F). Data are expressed as mean ± S.E.M. of three independent experiments conducted in duplicate.



**Supplemental Fig. S3.** Effect of preincubation of human liver cytosol with SERMs on LCA sulfonation. Human liver cytosol (200  $\mu$ g protein) was preincubated with PAPS (20  $\mu$ M) and a SERM (clomifene, tamoxifen, toremifene, ospemifene, droloxifene, raloxifene, arzoxifene, lasofoxifene, nafoxidine, bazedoxifene, or acolbifene; 10  $\mu$ M each), or vehicle (0.25% v/v methanol) at 37°C for 0, 30, 60, or 90 min. An aliquot (10  $\mu$ l) of the primary incubation mixture was incubated with PAPS (20  $\mu$ M) and LCA (2.5  $\mu$ M) for 30 min. Data are expressed as percentage of activity in the vehicle-treated control group that was not subjected to preincubation ( $29 \pm 2$  pmol/min/mg protein) and expressed as mean  $\pm$  S.E.M. for three independent experiments conducted in duplicate.