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The Stereoselective Sulfate Conjugation of 4-Methoxyfenoterol Stereoisomers by Sulfotransferase Enzymes

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Abstract

The presystemic sulfate conjugation of the stereoisomers of 4-methoxyfenoterol, (*R,R*)-MF, (*S,S*)-MF, (*R,S*)-MF, and (*S,R*)-MF, was investigated using commercially available human intestinal S9 fractions, a mixture of sulfotransferase (SULT) enzymes. The results indicate that the sulfation was stereospecific and that an *S*-configuration at the β -OH carbon of the MF molecule enhanced the maximal formation rates with (*S,R*)-MF > (*S,S*)-MF > (*R,S*)-MF $\approx$ (*R,R*)-MF, and competition studies demonstrated that (*S,R*)-MF is an effective inhibitor of (*R,R*)-MF sulfation (IC₅₀ = 60 μ M). In addition, the results from a cDNA-expressed human SULT isoform screen indicated that SULT1A1, SULT1A3, and SULT1E1 can mediate the sulfation of all four MF stereoisomers. Previously published molecular models of SULT1A3 and SULT1A1 were used in docking simulations of the MF stereoisomers using Molegro Virtual Docker. The models of the MF-SULT1A3 and MF-SULT1A1 complexes indicate that each of the two chiral centers of MF molecule plays a role in the observed relative stabilities. The observed stereoselectivity is the result of multiple hydrogen bonding interactions and induced conformational changes within the substrate-enzyme complex. In conclusion, the results suggest that a formulation developed from a mixture of (*R,R*)-MF and (*S,R*)-MF may increase the oral bioavailability of (*R,R*)-MF.

Keywords

sulfotransferases; SULT1A1; SULT1A3; SULT1E1; stereoselective phase II metabolism

INTRODUCTION

4-Methoxyfenoterol (MF) is a selective β_2 -adrenoceptor (β_2 -AR) agonist with two chiral centers and four stereoisomers, (*R,R*)-MF, (*R,S*)-MF, (*S,R*)-MF, and (*S,S*)-MF (Fig. 1). Recent studies have shown that the stereoisomer (*R,R*)-MF effectively stimulates cAMP

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accumulation in human embryonic kidney cells expressing β_2 -AR ($EC_{50} = 300$ nM), and contractility in a rat cardiomyocyte model system ($EC_{50} = 186$ nM),¹ inhibits mitogenesis in human-derived 1321N1 astrocytoma cells ($IC_{50} = 0.17$ nM), retards the growth of 1321N1 tumors implanted in the flank of SKID mice, and readily passes the blood–brain barrier.² These studies also demonstrated that the relative activities of the stereoisomers were (R,R) -MF $>$ (R,S) -MF $>$ (S,R) -MF $\gg$ (S,S) -MF.^{1,2} On the basis of these results, (R,R) -MF was chosen for development as a therapeutic agent in the treatment of congestive heart failure and β_2 -AR-expressing astrocytomas and glioblastomas.

(R,R) -MF is an analog of (R,R) -fenoterol ((R,R) -Fen) that is also a selective β_2 -AR agonist and that is currently in clinical trials for use in the treatment of congestive heart failure. The development of (R,R) -Fen as a single isomer drug represented a “racemic switch” as racemic (R,R,S,S) -Fen is marketed for the treatment of asthma.³ One of the problems associated with the use of (R,R,S,S) -Fen in humans is its oral bioavailability, which is only ~2%.⁴ The poor bioavailability of (R,R,S,S) -Fen is due to extensive presystemic sulfation by the sulfotransferase (SULT) enzymes.^{4,5}

Studies using the human-derived HepG2 cell line and recombinant SULT enzymes have demonstrated that (R,R,S,S) -Fen is conjugated by the phenolsulphotransferases P-PST (current nomenclature SULT1A1) and M-PST (SULT1A3).⁶ The SULT-mediated metabolism of Fen is regioselective and enantioselective, as SULT1A1 preferentially catalyzes sulfation at the 3,5-dihydroxyphenyl position and is selective for (S,S) -Fen, whereas SULT1A3 mediates sulfation at the 4-hydroxyphenyl position where it is highly selective for (R,R) -Fen and at the 3,5-dihydroxyphenyl position where it has a slight preference for (S,S) -Fen.⁶

On the basis of the regioselective and stereoselective sulfation of Fen, the 4-hydroxyphenyl position was converted to a 4-methylether with the objective of reducing presystemic sulfation and, thereby, increasing the oral bioavailability. The potential advantages of this approach were supported by the results from an initial study comparing the plasma profiles of (R,R) -Fen and (R,R) -MF obtained after i.v. and oral administration to the rat.⁷ The data demonstrated that after oral administration, (R,R) -MF had decreased clearance, increased half-life, and increased area under the curve relative to (R,R) -Fen and, after i.v. administration, a significantly higher systemic exposure. However, there is a significant species difference in the presystemic metabolism of (R,R) -Fen, as in the rat, Fen is primarily glucuronidated by UDP-glucuronosyltransferase enzymes as opposed to sulfation by SULT enzymes in humans.⁸

The current study was designed to investigate the *in vitro* sulfate conjugation of (R,R) -MF, (S,S) -MF, (R,S) -MF, and (S,R) -MF, the stereoselectivity of the sulfation, and the identity of the SULT enzymes that mediate the sulfation. The data indicate that an *S*-configuration at the β -OH carbon of the MF molecule enhances the maximal sulfation rates with (S,R) -MF $>$ (S,S) -MF (R,S) -MF $\approx$ (R,R) -MF, and competition studies demonstrated that (S,R) -MF is an effective inhibitor of (R,R) -MF sulfation ($IC_{50} = 60$ μ M). The results suggest that a formulation developed from a mixture of (R,R) -MF and (S,R) -MF may increase the oral bioavailability of (R,R) -MF. In addition, the results from a cDNA-expressed human SULT

isoform screen indicated that SULT1A1, SULT1A3, and SULT1E1 can mediate the sulfation of all four MF stereoisomers.

MATERIALS AND METHODS

Chemicals

(*R,R*)-MF, (*S,S*)-MF, (*R,S*)-MF, and (*S,R*)-MF were prepared as previously described as was [³H]-(*R,R*)-MF.^{9,10} Pooled human intestinal S9 protein, cytosolic extracts from *Escherichia coli* with individual recombinant human SULT isoforms (SULT1A1*1, SULT1A1*2, SULT1A2, SULT1A3, SULT1B1, SULT1C2, SULT1E1, and SULT2A1), and control cytosol were purchased from XenoTech LLC (Lenexa, KS, USA). Each recombinant isoform was tested for control sulfation activity with the respective probe substrate by XenoTech LLC, that is, 4-methylumbelliferone (SULT1A1*1, SULT1A1*2, and SULT1B1), 1-naphthol (SULT1A2 and SULT1A3), 4-nitrophenol (SULT1C2), and 17 β -estradiol (SULT2A1 and SULT1E1). Adenosine 3-phosphate 5-phosphosulfate (PAPS) (83% purity), dithiothreitol (DTT), bovine serum albumin (BSA), potassium phosphate monobasic, potassium phosphate dibasic, dimethyl sulfoxide (DMSO), ethyl nicotinate, formic acid, acetic acid, and hexyl nicotinate were purchased from Sigma (St. Louis, MO, USA). Acetonitrile (ACN) was purchased from Mallinckrodt (Phillipsburg, NJ, USA).

LC-MS/MS Separation and Identification of MF and MF Sulfate

The chromatography was carried out using a Shimadzu LC-20AD HPLC (Kyoto, Japan), and MS/MS analysis was performed using an Applied Biosystems 4000 QTRAP with a turbo ion spray interface in positive ion mode (AB Sciex, Foster City, CA, USA). MS/MS parameters included ion source temperature set at 500°C, ion spray voltage set at 3000 V, curtain gas flow set at 25 psi, and declustering potential set at 50 V. The data were acquired and processed using Analyst software v.1.4.2. Separation was achieved on a Luna C18(2) column (75 mm $\times$ 4.6 mm, 5 μ m) (Phenomenex, Torrance, CA, USA) with gradient elution at a constant flow rate of 1 ml/min using a mobile phase of 0.1% formic acid in water (solvent A) and 0.1% formic acid in ACN (solvent B). The gradient was started at 5% B until 1 min, increased to 60% B until 2.5 min, then 98% B until 2.8 min, held at 98% B until 5 min, then decreased to 5% B until 5.3 min. The total run time was 7 min. Each MF sulfate eluted at a consistent retention time of 2.8 min and was base-line separated from its respective MF isomer, which eluted at 2.6 min, and the retention time of ethyl nicotinate was 3.0 min. Data analysis was performed in multiple reaction monitoring mode with the following transitions: MF sulfate — *m/z*, 398 to 149, and ethyl nicotinate — *m/z*, 152 to 124. Ethyl nicotinate was used as an internal standard, and the MF sulfate/ethyl nicotinate peak area ratio was calculated for each sample.

Separation and Measurement of [³H]-(*R,R*)-MF and [³H]-(*R,R*)-MF Sulfate

[³H]-(*R,R*)-MF and [³H]-(*R,R*)-MF sulfate were separated using a Waters 2695 Separations Module (Waters Corporation, Milford, MA, USA) and a Packard Flow Scintillation Analyzer (Perkin Elmer, Waltham, MA, USA) using Ultima Gold scintillation cocktail (Perkin Elmer). Separation was achieved on a Luna C18(2) column (250 mm $\times$ 4.6 mm, 5 μ m) (Phenomenex) with gradient elution at a constant flow rate of 1 ml/min using a mobile

phase consisting of 0.1% formic acid in water (solvent A) and 0.1% formic acid in ACN (solvent B). The gradient started at 5% B and increased to 40% B until 4 min, remained at 40% B until 8 min, and then decreased to 5% B until 8.5 min, with a total run time of 10 min. The retention time was 5.1 min for [^3H]-(*R,R*)-MF and 6.2 min for [^3H]-(*R,R*)-MF sulfate.

Identification of Sulfate Metabolite of (*R,R*)-MF

Identification of the sulfated metabolites of (*R,R*)-MF was accomplished using LC-MS/MS analysis of sample extracts prepared from incubations of (*R,R*)-MF with human intestinal S9s. Final incubation conditions included 10 μM (*R,R*)-MF, 1 mg/ml intestinal S9 protein, 200 μM PAPS, 1 mM DTT, 0.05% BSA, 0.1% DMSO final in 0.1 M potassium phosphate buffer (pH 7.4) with an incubation time of 60 min. Incubations were initiated with the addition of PAPS and terminated with the addition of 0.5 volumes of ACN, vortexed briefly, then centrifuged at 18,000*g* for 5 min and analyzed by LC-MS/MS.

Sulfation of MF Stereoisomers by Human Intestinal S9 Fractions

Each of the four stereoisomers of MF was incubated with human intestinal S9 fractions to form the respective sulfates. The conditions of incubation, such as S9 protein and organic solvent (DMSO) concentrations, and incubation time were optimized in preliminary experiments by individually varying each parameter. Final incubation conditions included 1 mg/ml intestinal S9 protein, various concentrations of each isomer of MF (up to 250 μM , depending on the isomer), 200 μM PAPS, 1 mM DTT, 0.05% BSA, up to 1% DMSO in 0.1 M potassium phosphate buffer (pH 7.4) with an incubation time of 60 min. Incubations were terminated with the addition of 300 μl of 50 ng/ml ethyl nicotinate in ACN, vortexed briefly, and the samples were centrifuged at 18,000*g* for 5 min. The supernatants were transferred to a 96-well plate and stored at 4 $^{\circ}\text{C}$ until LC-MS/MS analysis.

K_m and maximal formation of MF sulfates were determined using Enzyme Kinetics ModuleTM 1.1 with Sigma Plot[®] 2001 (version 7.101, SPSS Inc., Chicago, IL, USA). The V_{max} of the reaction could not be estimated as a maximal velocity as pure standards of each sulfate were not available, and hence, each sulfate was not directly quantitated. Instead, the maximal formation of each sulfate has been reported as the ratio of the peak area of the sulfate versus the internal standard.

Screening of cDNA-expressed SULT Isoforms in the Sulfation of MF Stereoisomers

Human SULT isoforms expressed in *E. coli* (SULT1A1*1, SULT1A1*2, SULT1A2, SULT1A3, SULT1E1, and SULT2A1) and cytosol isolated from *E. coli* (negative control) were screened for MF sulfation. The concentrations used for the incubations were chosen on the basis of preliminary studies and were 50 and 100 μM for (*R,R*)-MF, 50 and 100 μM for (*S,S*)-MF, 40 and 80 μM for (*R,S*)-MF, and 30 and 60 μM for (*S,R*)-MF. The substrates were incubated with 0.1 mg/ml of each SULT isoform, control cytosol, or 1 mg/ml intestinal S9s, 200 μM PAPS, 1 mM DTT, 0.05% BSA, up to 1% DMSO in 0.1 M phosphate buffer (pH 7.4) with an incubation time of 60 min. Incubations were terminated with the addition of 300 μl of 50 ng/ml ethyl nicotinate in ACN, vortexed briefly, and the samples were then

centrifuged at 18,000g for 5 min. The supernatants were transferred to a 96-well plate and stored at 4°C until LC-MS/MS analysis.

Effect of (S,S)-MF, (R,S)-MF, and (S,R)-MF on the Sulfation of [³H]-(R,R)-MF

The effect of (S,S)-MF, (S,R)-MF, and (R,S)-MF on the human intestinal S9s-mediated sulfation of (R,R)-MF was determined using [³H]-(R,R)-MF as the substrate. Preliminary experiments were performed to select concentration ranges of each stereoisomer, and final incubation conditions included 50 μM (R,R)-MF (mixture of unlabeled and [³H]-labeled, with 0.2 μCi/ml), 1 mg/ml intestinal S9 protein, various concentrations (1 to 200 or 400 μM) of (S,S)-MF, (S,R)-MF, and (R,S)-MF, 200 μM PAPS, 1 mM DTT, 0.05% BSA, and 1% DMSO in 0.1 M potassium phosphate buffer (pH 7.4) with an incubation time of 60 min. Incubations were performed in a shaking water bath at 37°C and initiated with the addition of PAPS following a 5-min pre-incubation. Incubations were terminated with the addition of one volume of cold methanol, vortexed briefly, and the samples were centrifuged at 18,000g for 5 min. The supernatants were transferred to HPLC vials and analyzed for [³H]-(R,R)-MF and [³H]-(R,R)-MF sulfate using radio-chemical detection.

Docking Simulations

The four stereoisomers of MF were built in HyperChem software (6.03). Molegro Virtual Docker (MVD 2011.4.3.0) was employed to dock ligands into the binding cavity of enzyme models. Crystal structures of SULT1A3 (PDB: 1CJM, PDB: 2A3R), SULT1A1 (PDB: 1LS6), and SULT1E1 (PDB: 1G3M) were retrieved from Protein Data Bank. 2A3R. pdb structure (SULT1A3 containing the sulfate ion) was used to locate and to add the position of the SO₄²⁻ to the active sites of all enzyme models investigated in the docking experiments.

The locations of ligands natively co-crystallized in these structures were used to define binding cavities, whereas cofactors (PAP+ sulfate) and water molecules were explicitly taken into consideration in docking. The protonated form of each stereoisomer of MF was simulated in blind docking procedure with default settings of MVD package except of the number of runs and the number of results that were increased to 100 and 15, respectively. Docking results were visually inspected to find a pose in which the 3-OH moiety of MF was located in close proximity to a sulfate ion, allowing enzymatic reaction to occur. Visualization of results was made in Yasara (11.11.2).

RESULTS

Identification of Sulfate Metabolite of MF Stereoisomers

Because standards for the sulfate metabolite of (R,R)-MF were not available, the reaction product produced in the *in vitro* incubations of (R,R)-MF was identified using LC-MS/MS in positive and negative electrospray ionization modes. In positive and negative electrospray modes, the protonated (R,R)-MF (*m/z* = 318) and deprotonated (R,R)-MF (*m/z* = 316) and the ions of their corresponding monosulfate metabolite (*m/z* = 398 and *m/z* = 396) had similar fragmentation patterns as shown in the MS/MS mass spectra in Figure 2. The neutral loss experiment in LC-MS/MS further confirmed that the metabolite (MW = 397) can undergo neutral loss of SO₃, but not its parent compound (MW = 317).

Sulfation of MF Stereoisomers by Human Intestinal S9 Fractions

The presystemic sulfation of (*R,R*)-MF, (*S,S*)-MF, (*R,S*)-MF, and (*S,R*)-MF was investigated using commercially available intestinal S9 fractions, which is a mixture of both duodenal and jejunal fractions from the human small intestine. The kinetics of the sulfation of (*R,R*)-MF, (*S,S*)-MF, (*R,S*)-MF, and (*S,R*)-MF followed the Michaelis–Menten kinetics ($r^2 > 0.99$) (Fig. 3). The calculated Michaelis constants, K_m values, ranged from 52 μ M (*S,R*)-MF to 89 μ M (*S,S*)-MF, and the relative order of the stereoisomers was (*S,S*) > (*R,S*) $\approx$ (*R,R*) > (*S,R*) (Table 1). When the K_m values for the (*S,S*), (*S,R*), and (*R,S*) isomers were compared with the K_m values determined for (*R,R*)-MF, the only significant difference ($P < 0.01$) was found for (*S,S*)-MF.

Maximal formation rates of each sulfate ranged from 1.21 for (*R,R*)-MF sulfate to 3.70 for (*S,R*)-MF sulfate, and the relative order of the stereoisomers was (*R,R*) = (*R,S*) < (*S,S*) < (*S,R*) (Table 1). Statistically significant ($P < 0.01$) higher formation rates were found for the stereoisomers with an *S*-configuration at the β -OH carbon relative to those with an *R*-configuration at this chiral center, suggesting that the sulfation of MF is stereoselective, with maximal formation of (*R,R*)-MF sulfate and (*R,S*)-MF sulfate about 50% or lower than that of (*S,S*)-MF and (*S,R*)-MF. This was reflected in the enantioselectivity where the maximal formation of (*S,S*)-MF/(*R,R*)-MF was 2.0 and (*S,R*)-MF/(*R,S*)-MF was 2.9, whereas the diastereoselectivities of (*R,S*)-MF/(*R,R*)-MF and (*S,R*)/(*S,S*) were 1.0 and 1.5, respectively.

Screening of cDNA-expressed SULT Isoforms in the Sulfation of MF Stereoisomers

Because the S9 fraction is an undefined mixture of SULT enzymes, the source of the observed stereoselectivity was not evident from the initial data. Thus, the contribution of individual SULT isoforms to the *in vitro* sulfation of MF stereoisomers was studied using six human recombinant SULT isoforms expressed in *E. coli*, SULT1A1*1, SULT1A1*2, SULT1A2, SULT1A3, SULT1E1, and SULT2A1. Under the experimental conditions used in the study, SULT1A1*1, SULT1A1*2, SULT1A3, and SULT1E1 were capable of sulfation of all four stereoisomers of MF, whereas no significant sulfation was observed with SULT1A2, SULT1B1, SULT1C2, and SULT2A1 (Fig. 4).

Although the SULT1A1*1, SULT1A1*2, SULT1A3, and SULT1E1 isoforms mediated the sulfation of all of the MF stereoisomers, there were stereospecific differences between the relative contributions of these enzymes. Unlike the data from the experiments with the S9 fractions, the isoform specificity was due to the conformation at the chiral carbon on the aminoalkyl portion of MF. When the configuration at this site was *R*, SULT1A3-mediated sulfation was favored; the conjugation of (*R,R*)-MF and (*S,R*)-MF was ~1.5- to 2-fold greater than the sulfation by the other isoforms. When (*R,S*)-MF was the substrate, SULT1A1*1-catalyzed and SULT1A1*2-catalyzed sulfation was 1.5-fold greater than SULT1A3 and SULT1E1. When (*S,S*)-MF was the substrate, there were no significant differences between the SULT1A1*1-mediated, SULT1A1*2-mediated, and SULT1A3-mediated sulfation, whereas the activity of SULT1E1 was 1.3-fold lower. Notably, the sulfation rate of (*R,R*)-MF was significantly ($P < 0.0001$) lower than the rates observed with (*S,S*)-MF, (*R,S*)-MF, and (*S,R*)-MF in each of the SULT isoforms.

Effect of (*S,S*)-MF, (*R,S*)-MF, and (*S,R*)-MF on the Sulfation of [³H]-(*R,R*)-MF

The ability of (*S,S*)-MF, (*R,S*)-MF, and (*S,R*)-MF to inhibit the sulfate of (*R,R*)-MF was investigated using 50 μ M [³H]-(*R,R*)-MF as the substrate and the S9 fraction as the source of the SULT isoforms (Fig. 5, Supplemental Table 1). At equimolar concentrations, both (*R,S*)-MF and (*S,S*)-MF had little effect on the sulfation of (*R,R*)-MF, whereas (*S,R*)-MF reduced the conjugation by ~40%. These results are consistent with the calculated IC₅₀ values that were > 400 μ M (*R,S*)-MF, > 200 μ M (*S,S*)-MF, and ~60 μ M (*S,R*)-MF (Fig. 5). These results are also consistent with the data from the Michaelis-Menten kinetic studies where only the (*S,R*)-MF isomer had a lower K_m and higher maximal formation than (*R,R*)-MF (Table 1) and the expressed SULT isoform studies where SULT1A3-mediated sulfation was favored for the conjugation of (*R,R*)-MF and (*S,R*)-MF (Fig. 4).

Docking of MF Stereoisomer with SULT1A3 and SULT1A1

A number of crystal structures of sulfotransferases are deposited in RCSB Protein Data Bank.¹¹ On the basis of the experimental results described earlier, three molecular models, 2A3R.pdb (SULT1A3), 1LS6.pdb (SULT1A1), and 1G3M.pdb (SULT1E1), have been considered as targets for docking simulations of the four stereoisomers of MF using Molegro Virtual Docker. The locations of ligands natively co-crystallized with these enzymes were used to determine the binding cavities and to define the correct position of the hydroxyl group to be sulfated. Although 2A3R and 1G3M models appeared very effective in docking simulations and results interpretations, the 1G3M model, representing SULT1E1 subtype, produced inconsistent and questionable docking results. One possible explanation is that the crystal structure did not contain coordinates of two residues essentially located within the substrate binding cavity (Lys85 and Met90; see supporting materials). Because of the uncertainty of location of these residues, docking simulations to the SULT1E1 subtype model are not presented and further discussed.

The MolDockScore values obtained in docking of the four stereoisomers of MF into SULT1A1 and SULT1A3 (Table 2). The results obtained on both enzyme isoforms indicate that the scoring values are the lowest for (*S,R*)-MF relative to the other MF stereoisomers and the highest for (*R,S*)-MF, whereas the other two enantiomers, (*R,R*)-MF and (*S,S*)-MF, have their scoring values in between (Table 2).

The models of the MF-SULT1A3 complexes indicate that these complexes are stabilized by a network of hydrogen bonds between the ligands and the enzyme; some of these interactions are common to all of the stereoisomers. The putative binding mode is represented in Figure 6A using (*S,R*)-MF. Hydrogen bonding includes interactions between the 3-OH substituent on a MF molecule and Lys106 and His108 moieties on the SULT1A3, depicted by the green arrows in Figure 6A. Both hydrogen bonds position the oxygen atom of the 3-OH moiety for the strong electronegative interaction with PAPS, depicted by the yellow arrow in Figure 6A. These and one additional hydrogen bond between the 5-OH moiety and the His149 residue are common to all stereoisomers in complex with SULT1A3 model. But Figure 6A contains three other hydrogen bonds simulated for (*S,R*)-MF isomer: the 4-methoxy moiety and the Thr95 residue, the β -hydroxy and the protonated amino group that interact with the Asp86 residue.

The models as well as MolDockScore values provide an explanation for the role of stereoconfiguration of each chiral center of MF molecule in complex with SULT1A3. When the configuration of the β -carbon atom is switched from *S* to *R*, the β -hydroxy group reorients and forms a hydrogen bond with adjacent Glu146 residue as illustrated by the complex (*R,R*)-MF-SULT1A3 (Fig. 6B). As prompted by scoring values, such distributed interaction pattern is slightly less stable than the double interaction of Asp86 with both amino and β groups as observed for the (*S,R*)-MF-SULT1A3 complex. But simulations suggest that more important for the recognition of isomers is the stereoconfiguration at the chiral center of the aminoalkyl portion of MF. The *R*-configuration fits to the steric requirements of the binding cavity that compels the molecule to undergo significant conformational adjustments in order to reach Thr95 for an interaction with its 4-methoxy moiety. As a result, the (*S,S*)-MF loses this interaction, preserving all others observed in (*S,R*)-MF, whereas the (*R,S*)-MF loses an interaction not only between 4-OH and Thr86 but also between amino moiety and Asp86. The latter explains why the (*R,S*)-MF-SULT1A3 complex is the least stable in docking simulations.

Figure 7 presents results of MF stereoisomers docking to the SULT1A1 model. The putative binding mode is represented in Figure 7 using (*S,R*)-MF. Noticeably, the molecule forms fewer hydrogen bonding interactions with SULT1A1 than observed in SULT1A3 docking. The 3-OH substituent is engaged in hydrogen bonding interactions with the Lys106 and His108 residues and electrostatic interaction with PAPS; the 5-OH and 4-OMe moieties interact with His149 and Thr95, respectively. But a major difference between SULT1A1 and SULT1A3 isoforms is that because of protein sequence differences, the binding cavity of the SULT1A1 contains two Ala residues in positions 86 and 146, instead of the acidic Asp86 and Glu146 residues present in the SULT1A3 sequence (Supplemental Figure 1). Therefore, no additional hydrogen bond interactions are found between SULT1A1 protein and protonated amino and β -OH moieties of the MF molecule (Fig. 7). This time, van der Waals interactions and the steric fit seem to play greater roles in the binding of this fragment of MF molecule.

In spite of the sequence differences, the trend of scoring values is similar to that observed in SULT1A3 docking (Table 2). Again, the lowest MolDockScore value was calculated for (*S,R*)-MF and the highest for (*R,S*)-MF isomer. Noticeably, the value for (*S,S*)-MF is significantly lower than for (*R,R*)-MF, which was not the case in SULT1A3 modeling. It underlines possibly an even greater role of the second chiral center in the stereoisomer recognition. The docking indicates that the MF stereoisomers with the *S*-configuration conform best to the steric requirements of the SULT1A1 binding cavity. The results further suggest that the molecular conformation of the (*S,x*)-MF isomers is also favored by the SULT1A1 isoform.

DISCUSSION

The SULT enzyme family mediates the sulfate conjugation of a number of endogenous and exogenous compounds and plays a role in the bioavailability of a number of drug substances.^{6,12,13} Thus, the determination of the effect of SULT isoforms on the bioavailability of a drug candidate is an important step in the drug development process. This is of particular

importance for (*R,R*)-MF, which is being developed for potential use in congestive heart failure and in the treatment of glioblastomas^{1,2} (*R,R*)-MF is a derivative of the β_2 -adrenoceptor agonist (*R,R,S,S*)-Fen. Previous studies have demonstrated that the SULT-mediated conjugation of (*R,R,S,S*)-Fen occurs at both the 3,5-dihydroxyphenyl and 4-hydroxyphenyl positions and that the sulfation is enantiospecific and regioselective.^{6,12} In these studies, the potential for sulfation at the 4-hydroxyphenyl position was blocked and the sulfation of all four stereoisomers of MF at the 3,5-dihydroxyphenyl position investigated. The data are consistent with previous studies of (*R,R,S,S*)-Fen and demonstrate that the sulfation of MF stereoisomers by human intestinal S9 fractions was enantiospecific and stereospecific with a relative maximal sulfate formation of (*S,R*)-MF > (*S,S*)-MF > (*R,R*)-MF = (*R,S*)-MF (Table 1). Competitive sulfation studies have also demonstrated that (*S,R*)-MF can effectively inhibit the sulfation of (*R,R*)-MF, as a 2:1 ratio of (*S,R*)-MF/(*R,R*)-MF resulted in a 61% reduction in the production of (*R,R*)-MF sulfate (Fig. 5, Supplemental Table 1). Because (*S,R*)-MF has less than 5% of the β_2 -AR agonist activity of (*R,R*)-MF (unpublished data), the results of this study suggest that the co-administration of (*S,R*)-MF may represent an approach to the reduction of the presystemic sulfation of (*R,R*)-MF, resulting in increased bioavailability. Unequal isomer formulations of (*S,R*)-MF: (*R,R*)-MF in ratios of 2:1 and 3:1 have been prepared, and the effect of the oral administration of these mixtures on the bioavailability of (*R,R*)-MF will be reported elsewhere.

Previous studies have demonstrated that there is a high interindividual variability in SULT enzyme expression and activity, which has been attributed to both environmental and genetic factors.¹⁴ For example, SULT1A1 exhibits over 50-fold variations in enzyme activity, associated in part with single nucleotide polymorphisms and copy number variations.¹⁵ Thus, it is important to identify the contributions of the individual SULT isoforms to the sulfation of MF stereoisomers and the potential for pharmacogenetic differences in bioavailability. The data from this study demonstrate that the sulfation of MF stereoisomers is mediated by SULT1A1*1, SULT1A1*2, SULT1A3, and SULT1E1 isoforms. These results are consistent with the initial studies of the isoform-specific sulfation of (*R,R,S,S*)-Fen in which SULT1A1 and SULT1A3 were identified as the key mediators in the conjugation at the 3,5-dihydroxyphenyl position.⁶

The data from this study also demonstrate that the sulfation of MF is stereospecific (Fig. 4). When the configuration at this site was *R*, SULT1A3-mediated sulfation was favored as the conjugation of (*R,R*)-MF, and (*S,R*)-MF was ~1.5- to 2-fold greater than the sulfation by the other isoforms. When (*R,S*)-MF was the substrate, SULT1A1*1-catalyzed and SULT1A1*2-catalyzed sulfation was 1.5-fold greater than SULT1A3 and SULT1E1. When (*S,S*)-MF was the substrate, there were no significant differences between the SULT1A1*1-mediated, SULT1A1*2-mediated and SULT1A3-mediated sulfation, whereas the activity of SULT1E1 was 1.3-fold lower. These results are consistent with the data from the incubations with the S9 human intestinal fractions and support the use of (*S,R*)-MF as the competitive inhibitor of the presystemic sulfation of (*R,R*)-MF. The results are also consistent with the previous studies of (*R,R,S,S*)-Fen in which SULT1A1 preferentially catalyzed the sulfation of (*S,S*)-Fen, whereas SULT1A3 had a slight preference for (*S,S*)-Fen relative to (*R,R*)-Fen.⁶

The source of the observed stereospecificity was also explored using molecular models of the SULT1A1 and SULT1A3 isoforms. The results indicate that the molecular bases of stereospecificity of the enzymes reflected by the docking results are consistent with experimentally determined stereospecificity, as the (*S,R*)-MF-SULT complexes formed with SULT1A1 and SULT1A3 had the lowest MolDocScore values. When the MF stereoisomers were docked in the SULT1A3 and SULT1A1 enzymes in the position associated with the sulfation of the 3-OH group, the modeling shows that all of the complexes were stabilized by hydrogen-bonding interactions between the 3-OH substituent on the MF molecule and Lys106 and His108 residues on the SULT molecule and between the 5-OH moiety and the His149 residue. The results also indicate that the configuration of both of the chiral centers plays a role in the stability of this substrate–enzyme complex, as the steric requirements of the SULT1A1 and SULT1A3 active sites require the substrate to undergo conformational changes to fit into this site. The data from the modeling studies indicate that the MF stereoisomers with the *S*-configuration at the chiral center on the aminoalkyl conform best to these steric requirements. The data also indicate that in the MF-SULT1A3 complexes, (*S,R*)-MF forms additional hydrogen bonds between the 4-methoxy moiety and the Thr95 residue, the β -hydroxy and the protonated amino group, and the Asp86 residue. When the configuration of the β -carbon atom is switched from *S* to *R*, the β -hydroxy group reorients and forms a hydrogen bond with the adjacent Glu146 residue that is slightly less stable than the double interaction of Asp86 with both amino and β -groups. This produces the difference in the relative stabilities where the (*S,R*)-MF-SULT1A3 complex has a lower MolDocScore than the (*R,R*)-MF-SULT1A3 complex.

The data from this study suggest that the models utilized in the docking experiments will be useful in the design of new Fen derivatives that are not substrates of the SULT enzymes and, therefore, more readily bioavailable. This possibility is being explored using the previously reported fenoterol analogs, which represent a broad range of structural and stereochemical probes.^{1,2} The results of these studies will be reported elsewhere.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Scheme of sulfation of (*R,R*)-4-methoxyfenoterol ((*R,R*)-MF). PAPS, adenosine 3-phosphate 5-phosphosulfate.

Fig. 2. MS/MS spectra of (*R,R*)-MF (**A** and **C**) and its monosulfate (**B** and **D**) in positive (**A** and **B**) and negative (**C** and **D**) electrospray ionization mode.

Fig. 3. Kinetics of (*R,R*)-MF (A), (*S,S*)-MF (B), (*R,S*)-MF (C), and (*S,R*)-MF (D) sulfation by human intestinal S9 fractions. Each data point represents the mean of three determinations ($\pm$ SD).

Fig. 4. Role of specific cDNA-expressed sulfotransferase (SULT) isoforms on the sulfation of (*R,R*)-MF (**A**), (*S,S*)-MF (**B**), (*R,S*)-MF (**C**), and (*S,R*)-MF (**D**). Each bar represents the mean of three determinations ($\pm$ SD).

Fig. 5. Effect of (S,S)-MF, (R,S)-MF, and (S,R)-MF on the sulfation of [3H]-(R,R)-MF by human intestinal S9 fractions. Each data point represents the mean of three determinations ($\pm$ SD).

Fig. 6. Docking pose of (A) (*S,R*)-MF and (B) (*R,R*)-MF obtained in docking to the enzymatic binding site model of SULT1A3 protein. Green arrows show hydrogen bonds identified in the complex, and yellow arrows indicate the interaction with adenosine 3-phosphate 5-phosphosulfate (rendered as SO_3^- in yellow ball mode). Substrate molecules and essential residues are shown explicitly in element color-coded stick mode, all non-polar hydrogens are hidden. The only significant difference between two diastereoisomer conformation in complex is the reorientation of the β -OH moiety and hydrogen bond formation with either ASP86 residue in (*S,R*)-MF or Glu146 residue in (*R,R*)-MF complex.

Fig. 7. Docking pose of (*S,R*)-MF obtained in docking to the enzymatic binding site model of SULT1A1 protein. Green arrows show hydrogen bonds identified in the complex, and the yellow arrow indicates the interaction with adenosine 3-phosphate 5-phosphosulfate (rendered as SO_3^- in yellow ball mode). Substrate molecules and essential residues are shown explicitly in element color-coded stick mode, all non-polar hydrogens are hidden.

TABLE 1

The kinetics of the sulfation of the stereoisomers of 4-methoxyfenoterol (MF) using human intestinal S9 fractions, where K_m is the Michaelis constant

Parameter	(<i>R,R</i>)-MF	(<i>S,S</i>)-MF	(<i>R,S</i>)-MF	(<i>S,R</i>)-MF
K_m (μM , mean $\pm$ SE)	61.99 ± 1.27	$89.21^a \pm 2.59$	68.55 ± 3.20	51.98 ± 3.36
Maximal formation of sulfate (peak area ratio of sulfate/int std)	1.21 ± 0.01	$2.47^a \pm 0.03$	1.25 ± 0.03	$3.70^a \pm 0.11$

^aSignificantly ($p < 0.01$) higher than the corresponding value with *R,R*-MF (unpaired *t*-test).

TABLE 2

Comparison of MolDockScore values obtained in docking of MF stereoisomers to SULT1A3 and SULT1A1 models

	(<i>R,R</i>)-MF	(<i>R,S</i>)-MF	(<i>S,R</i>)-MF	(<i>S,S</i>)-MF
SULT1A3	−129.077	−126.106	−132.899	−128.465
SULT1A1	−130.828	−128.317	−142.913	−139.051

MF, 4-methoxyfenoterol; SULT, sulfotransferase.