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Enantioselective inhibition of Cytochrome P450-mediated drug metabolism by a novel antithrombotic agent, S002-333: Major effect on CYP2B6

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ABSTRACT

A significant number of new chemical entities (NCEs) fail in drug discovery due to inhibition of Cytochrome P450 (CYP) enzymes. Therefore, to avert costly drug failure at the clinical phase it becomes indispensable to evaluate the CYP inhibition profile of NCEs early in drug discovery. In light of these concerns, we envisioned to investigate the inhibitory effects of S002-333 [2-(4-methoxy-benzenesulfonyl)-2,3,4,9-tetrahydro-1H-b-carboxylic acid amide], a novel and potent antithrombotic agent, on nine major CYP enzymes (CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4) of human liver microsomes (HLM). S002-333 exists as racemic mixture of S004-1032 (*R*-isomer) and S007-1558 (*S*-isomer), consequently, we further examined the enantioselective differences of S002-333 in the inhibition of human CYP enzymes. Of the CYP enzymes tested, CYP2B6-catalyzed bupropion 6-hydroxylation was inhibited by S002-333 (IC_{50} ~ 9.25 ± 2.46 μM) in a stereoselective manner with (*S*)-isomer showing potent inhibition (IC_{50} ~ 5.28 ± 1.25 μM) in contrast to (*R*)-isomer which showed negligible inhibition on CYP2B6 activity (IC_{50} > 50 μM). S002-333 and its (*S*)-isomer inhibited CYP2B6 activity in a non-competitive fashion with estimated K_i values of 10.1 ± 3.4 μM and 5.09 ± 1.05 μM, respectively. No shift in the IC_{50} value was observed for S002-333 and its isomers when preincubated for 30 min in the presence of NADPH suggesting that neither S002-333 nor its enantiomers are time-dependent inhibitors. Thus, the present findings signified that S002-333 is a potent stereoselective inhibitor of CYP2B6, whereas, inhibition for other CYPs was substantially negligible. These *in vitro* findings would be useful in deciding the development of S002-333 as a single-enantiomer or as a racemic mixture.

1. Introduction

Antithrombotic drugs are widely used for the prevention and treatment of cardiovascular ailments associated with intravascular disorders. The inability of the commonly prescribed antithrombotic drugs such as aspirin and/or clopidogrel to fully suppress the platelet activation stimulus at the site of platelet disruption, development of resistance to the antiplatelet effect and the risk of excessive bleeding associated has prompted the researchers to develop new antithrombotic drugs [1,2]. In the pursuit of safe and effective antithrombotic agents, S002-333 [2-(4-methoxy-benzenesulfonyl)-2,3,4,9-tetrahydro-1H-b-carboxylic acid amide] was developed by CSIR-Central Drug Research Institute (CSIR-CDRI), India, for the treatment of intravascular thrombosis such as myocardial ischemia and stroke (Patent granted: WO2006070385 A1). It exhibited marked antithrombotic activity in collagen- and epinephrine-induced thrombosis in mice and reduced collagen-mediated human platelet aggression. It also exhibited promising profile against venous thrombosis model in rabbits and displayed minimal danger of bleeding or unrelated side effects in comparison to standard therapies such as aspirin and clopidogrel in mouse model [3]. However, S002-333 is defined as the racemic mixture of

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two enantiomers: S004-1032 (*R*-isomer) and S007-1558 (*S*-isomer) [4]. It is well recognized that enantiomers of a chiral drug may differ in their pharmacokinetic properties such as metabolism, CYP inhibition, excretion, selectivity for receptors, transporters and/or enzymes and toxicity [5]. In such cases, it becomes very crucial to determine the safety and efficacy of the racemic mixture and its individual isomers in early stage of drug discovery since it would assist in deciding whether the racemic mixture or single enantiomer should be considered as a lead for further development.

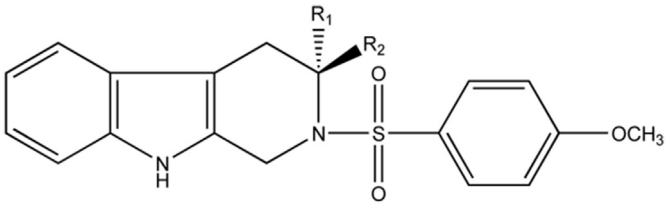
Thorough investigation of the drug-drug interaction (DDI) potential of a new drug must be carried out before it can be marketed. Over the past decades, CYP enzyme inhibition has been considered to be the most common cause of harmful DDIs which has even led to the expulsion of various new drugs from the market [6]. The proportion of inhibition and induction of CYP is 70% and 30%, respectively, in CYP-mediated DDIs [7]. Consequently, it is essential to evaluate the inhibitory effect of a drug candidate on CYP enzymes to predict the need and/or designing the *in vivo* clinical trials. A great deal of attention should be paid in comparing the inhibitory effects of enantiomers on P450-mediated metabolism *in vitro* since stereoselectivity in the inhibition of CYP-mediated metabolism by many chiral drugs has been widely reported. For instance, *S*-(−)-reduced haloperidol showed a ten-fold lower K_i for CYP2D6 inhibition in comparison to *R*-(+)-enantiomer [8]; *S*-(+)-ketoprofen had 13 times more potent inhibition of cyclooxygenase activity than *R*-(−)-ketoprofen [9]; and CYP3A4-mediated testosterone and methadone metabolism was inhibited twice by L-ketoconazole than D-ketoconazole [10].

Stereoselectivity in the metabolism of S002-333 by recombinant human P450s and human liver microsomes (HLM) has been reported earlier. S002-333 and its (*S*)-isomer were predominantly metabolized by CYP2C19 whereas the (*R*)-isomer was majorly metabolized by CYP3A4 [11]. Hence, it is anticipated that there might be some possibility of stereoselectivity in inhibiting the CYP-mediated drug metabolism by S002-333. Bearing this in mind, we examined the inhibitory potential of S002-333 and its enantiomers on US FDA recommended nine CYP enzymes (CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4) of HLM. Full-scale characterization of mechanism of inhibition (reversible and/or irreversible) was investigated for those showing potent inhibition ($IC_{50} \leq 10$ μM) [12]. In addition, IC_{50} shift assay was conducted to determine whether S002-333 and its enantiomers are time-dependent inactivators of CYP2B6. The outcomes of the present investigation would provide the inhibitory properties of the S002-333 and its enantioselective differences in the inhibition of human CYP enzymes.

2. Materials and methods

2.1. Chemicals and reagents

S002-333 and its isomers, S004-1032 (*R*-isomer) and S007-1558 (*S*-isomer) (purity >99%) (Fig. 1) were synthesized and purified at Medicinal Process Chemistry Division of CSIR-CDRI. Phenacetin, acetaminophen, coumarin, 7-hydroxycoumarin, bupropion hydrochloride, hydroxybupropion, paclitaxel, 6α-hydroxypaclitaxel, omeprazole, 5-hydroxyomeprazole α-Naphthoflavone, pilocarpine hydrochloride, ticlopidine hydrochloride, N-benzylnirvanol, rosuvastatin (internal standard 1, IS1) and chlorthalidone (internal standard 2, IS2), formic acid, trizma base and magnesium chloride were purchased from Sigma-Aldrich (St. Louis, MO, US). Diclofenac sodium, 4'-hydroxydiclofenac, chlorzoxazone, 6-hydroxychlorzoxazone, midazolam, 1'-hydroxymidazolam, dextromethorphan hydrobromide, dextrorphan tartrate, quercetin, sulfaphenazole, quinidine, ammonium diethyldithiocarbamate and



Enantiomers of S002-333	R_1	R_2
S004-1032 (*R*-isomer)	H	$CONH_2$
S007-1558 (*S*-isomer)	$CONH_2$	H

Fig. 1. Chemical structures of the enantiomers of S002-333.

ketoconazole were purchased from Cayman Chemicals (Ann Arbor, MI, US). β-nicotinamide adenine dinucleotide phosphate (NADPH) was purchased from SRL (Mumbai, India). Pooled human liver microsomes were purchased from BD Biosciences (San Jose, CA, US). Chromatographic grade methanol and acetonitrile (ACN) were obtained from Merck Chemicals (Darmstadt, Germany). Ultrapure water was obtained in-house using a Milli-Q PLUS PF water purifying system (Millipore, Bedford, MA). All other reagents and solvents were of analytical grade and purchased from standard chemical suppliers.

2.2. Screening of reversible inhibitory effects of S002-333 and its enantiomers on nine CYP activities

Recently, the use of "cocktail" assay has gained popularity since it utilizes the mixture of CYP probe substrates to assess the inhibition of several CYP enzymes simultaneously [13,14]. Therefore, inhibitory potency of S002-333 and its enantiomers were determined using the previously reported method with slight modifications [15,16]. Phenacetin O-deethylase, coumarin 7-hydroxylase, bupropion 6-hydroxylase, paclitaxel 6α-hydroxylase, diclofenac 4-hydroxylase, omeprazole 5-hydroxylase, dextromethorphan O-demethylase, chlorzoxazone 6-hydroxylase, and midazolam 1'-hydroxylase activities were determined as probe activities in HLM for CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4, respectively. The standard solutions of each substrate were prepared in methanol, except coumarin (prepared in ACN) and diluted serially in methanol to obtain the required concentrations. Incubation conditions specific for each substrate were optimized by carrying out preliminary experiments to determine linearity with respect to time, substrate concentration and protein concentration [17]. The final concentration of substrate used in the incubation mixture was less than or equal to their respective Michaelis constant (K_m) as shown in the Table 1 [18,19] and the concentration range used for test compounds: S002-333, (*R*)-isomer and (*S*)-isomer was 0.026–100 μM. Positive control experiments were conducted in the presence of enzyme-specific standard inhibitors (concentrations are shown in Table 2).

All the incubations were performed in triplicate each in 100 μL reaction medium containing tris buffer (50 mM, pH 7.4), $MgCl_2$ (10 mM), HLM (0.2 mg/mL), test compound and CYP substrate cocktail at 37 °C. The final organic solvent concentration used in the microsomal assay did not exceed 0.5% (v/v). Following pre-incubation for 10 min, the reaction was initiated by the addition of NADPH at a final concentration of 4 mM and incubated for 15 min. Subsequently, the reaction was terminated by protein precipitation using ice-cold ACN containing IS1 and IS2 to facilitate the extraction