ABSTRACT — The relative contribution of cytochrome P450 (CYP) isoforms responsible for carvedilol (CAR) oxidation in rats were evaluated in order to compare with that of reported human CYPs responsible for the metabolism of CAR enantiomers. The depletion of CAR enantiomers by recombinant CYPs and the effects of CYP-selective inhibitors on the depletion catalyzed by rat liver microsomes (RLM) was determined. Quinine (rat CYP2D inhibitor) markedly inhibited the metabolism of both R- and S-CAR by RLM. The metabolism of S-CAR was inhibited more than that of R-CAR by furafylline, (a CYP1A2 inhibitor, 53.5% vs 11.3%), α-naphthoflavone (a CYP1A2 inhibitor, 64.5% vs 33.6%), and ketoconazole (a CYP3A inhibitor, 87.1% vs 51.2%). Among the CYPs examined, CYP2D2 showed the highest metabolic activities against both the enantiomers. R-CAR was mainly metabolized by CYP2D2 and CYP3A2. CYP2C11 and CYP3A1, in addition to CYP2D2 and CYP3A2 showed higher metabolic activities against S-CAR than that against R-CAR. These results suggest that CYP2D2 predominantly catalyzed R-CAR metabolism, whereas CYP2D2 and CYP3A1/2 catalyzed S-CAR metabolism in rats.

Key words: Chirality, CYP enzymes, Cytochrome P450, Metabolism

INTRODUCTION

Carvedilol (CAR), a third-generation β-adrenoceptor antagonist which causes not only β-blockage but also α1-blockage (vasodilatation) (Fisker et al., 2015), is used clinically as a racemic mixture of R- and S-CAR. CAR possesses both enantioselective pharmacodynamic and pharmacokinetic properties (Neugebauer et al., 1987, 1990; Zhou and Wood, 1995; Stoschitzky et al., 2001). Although there is no difference regarding the α-blocking specific activity of the enantiomers, the β-blocking specific activity of S-CAR is stronger than that of R-CAR (Stoschitzky et al., 2001; Bartsch et al., 1990). In humans, the clearance of R-CAR is lower than that of S-CAR (Neugebauer et al., 1990; Zhou and Wood, 1995), and R-CAR is mainly metabolized to form 4′- and 5′-hydroxy-CAR by polymorphic cytochrome P450 (CYP)2D6 (Oldham and Clarke, 1997). Although the clearance of R-CAR is further reduced in poor metabolizers (PMs) of CYP2D6 (Neugebauer et al., 1987, 1990), considerable CAR metabolism was observed in the livers of PMs (Neugebauer et al., 1987; Zhou and Wood, 1995; Oldham and Clarke, 1997), suggesting that CAR is stereoselectively metabolized in humans, and other CYP isoforms contribute to this metabolism. In fact, CYP2D6, CYP2C9, CYP2E1, CYP1A2, and CYP3A4 oxidize CAR to 4′-, 5′-, and 8-hydroxy, and O-desmethyl metabolites (Oldham and Clarke, 1997). Because the two enantiomers of CAR differ in their pharmacological and pharmacokinetic properties in humans, it is important to identify the individual CYP isof orm(s) involved in their metabolism. Genetic polymorphisms of CYP such as CYP2D affect the drug metabolism and disposition and result in large interindividual variations in pharmacokinetics of drugs. The smaller interindividual variation in drug exposure is preferable to evaluate drug toxicity. Therefore, since the genetic polymorphism of CYP2D demonstrated in human was also reported in rats, assessment of the contribution of CYP isof orm including genetic polymorphism enzymes to CAR metabolism is important in rats. There are some reports on the toxicity of CAR. For example, CAR facilitates doxorubicin cytotoxicity via inhibition of P-glycoprotein activity (Jonsson et al., 1999). These toxicities are possibly asso-
associated with the metabolism and disposition of CAR. Rats and dogs are used in the toxicity test of CAR (interview form for CAR (Artist® tablets), http://www.info.pmda.go.jp/go/interview/2/430574_2149032F1021_2_A15_1F). There are genetic polymorphisms of CYP isoforms metabolizing CAR. For example, CYP2D6 which is highly polymorphic enzyme (> 100 allelic variants) are involved in CAR metabolism. Therefore, the present study aimed to clarify the CYP isoforms involving in R-CAR and S-CAR metabolism and to compare it to the previous results in human liver microsome (Iwaki et al., 2016).

We recently demonstrated the quantitative characterization of the human CYP isoforms responsible for the stereoselective biotransformation of CAR by measuring the disappearance rate of parent compounds (Iwaki et al., 2016). In the preclinical development of new drugs, pharmacokinetic and metabolic properties, and toxicological properties are determined in animals such as rats. There is little information available on the quantitative contribution of each CYP isoform to R- and S-CAR metabolism in rats. Thus, we investigated the quantitative characterization of the rat CYP isoforms responsible for the stereoselective metabolism of CAR.

MATERIALS AND METHODS

Materials
Recombinant rat CYP enzymes (CYP1A2, CYP2C11, CYP2C12, CYP2D1, CYP2D2, CYP3A1, and CYP3A2) expressed in baculovirus-infected insect cells, insect cell control microsomes, and pooled 168-donor Sprague Dawley male rat liver microsomes (RLM) were purchased from BD Gentest (Wobum, MA, USA) and BD Biosciences Discovery Labware (Billerica, MA, USA), respectively. R-(+)- and S-(−)-CAR were obtained from Toronto Research Chemicals (Toronto, Canada). Propranolol hydrochloride, as an internal standard for high-performance liquid chromatography (HPLC) analysis, furafylline, α-naphthoflavone, and quinine were obtained from Sigma Aldrich (St. Louis, MO, USA). Ketoconazole was purchased from BD Gentest. NADP+, glucose-6-phosphate (G6P), and glucose-6-phosphate dehydrogenase (G6PDH) were purchased from Oriental Yeast (Tokyo, Japan). All other chemicals used were of the highest purity available.

HPLC assay of CAR
The concentrations of R- and S-CAR were measured using an HPLC system (Liquid Chromatograph model LC-10AD; Shimadzu, Kyoto, Japan) equipped with a fluorescence detector (RF-10A), according to the method reported by Rathod et al. (2007) with some modifications (Iwaki et al., 2016). Briefly, chromatographic separation was achieved using a Cosmosil 5C18-ARII column (4.6 × 150 mm, 5 μm, Nacalai Tesque, Kyoto, Japan) maintained at 40°C. The mobile phase consisted of 20 mM phosphate buffer, pH 6.8/methanol/triethylamine (35:65:0.1, v/v/v). The flow rate was maintained at 1.0 mL/min. The excitation and emission wavelengths were set at 280 nm and 330 nm, respectively. Standard curves for R- and S-CAR were linear over the concentration range of 0.01-10 μM. The minimum quantifiable concentration of R- and S-CAR was 0.01 μM.

Statistical analysis
The data are expressed as the mean ± standard deviation (S.D.). The difference in the mean between the groups was analyzed statistically using Dunnett’s test after an analysis of variance. GraphPad Prism 6 (GraphPad...
RESULTS

Chemical inhibition of CAR metabolism by RLM

The effects of various chemical inhibitors, such as furafylline and α-naphthoflavone (CYP1A2 inhibitors), quinine (rat CYP2D inhibitor), and ketoconazole (CYP3A inhibitor), on the metabolism of R- and S-CAR by RLM (Fig. 1, Table 1) were investigated. The metabolism of both R- and S-CAR by RLM were markedly inhibited by 1 μM quinine (more than 71.6% inhibition). On the contrary, the metabolism of S-CAR was more inhibited than that of R-CAR by 25 μM furafylline (53.5% vs 11.3%), 50 μM α-naphthoflavone (64.5% vs 33.6%), and 1 μM ketoconazole (87.1% vs 51.2%). There were no significant differences between K_{dep} in quinine and K_{dep} in ketoconazole.

Depletion of CAR by recombinant CYPs

The depletion profiles of R- and S-CAR at various substrate concentrations of main CYPs, such as CYP1A2, CYP2C11, CYP2C12, CYP2D1, CYP2D2, CYP3A1, and CYP3A2, are shown in Fig. 2. Among the CYPs examined, CYP2D2 showed the highest metabolic activities against both the enantiomers. R-CAR was mainly metabolized by two major CYP isoforms, CYP2D2 and CYP3A2, whereas CYP2C11 and CYP3A1 in addition to CYP2D2 and CYP3A2 showed higher metabolic activities against S-CAR. The activity of each CYP against S-CAR was higher than that against R-CAR, except for...

Table 1. Comparison of CYPs that contribute to the metabolism of CAR enantiomers in RLM and HLM.

<table>
<thead>
<tr>
<th></th>
<th>Inhibition in RLM (%) a)</th>
<th>Contribution in HLM (%) b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R-CAR</td>
<td>S-CAR</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>11.3-33.6</td>
<td>53.5-64.5</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CYP2C11</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>CYP2C12</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CYP2D1/2</td>
<td>71.6</td>
<td>78.0</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CYP3A2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>51.2</td>
<td>87.1</td>
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a) Inhibitor: furafylline (25 μM) or α-naphthoflavone (50 μM) for CYP1A2, quinine (1 μM) for CYP2D1/2, and ketoconazole (1 μM) for CYP3A2 (see Fig. 1). b) Contribution in HLM (%) estimated by using recombinant human CYP was mentioned in our previous report (Iwaki et al., 2016). NA: not applicable. Bold: the highest inhibition or contribution in RLM or HLM, respectively.
CYP3A2 and CYP2C12.

DISCUSSION

In our previous study, the quantitative contribution of different CYP isoforms to enantioselective CAR metabolism was evaluated using a substrate depletion assay in humans (Iwaki et al., 2016). The study results indicated that CYP2D6 shows the highest contribution to the metabolism of the R-enantiomer, followed by CYP3A4, CYP1A2, and CYP2C9, whereas the metabolism of the S-enantiomer is mainly mediated by CYP1A2, followed by CYP2D6 and CYP3A4 (Table 1).

In rats, R-CAR metabolism was found to be catalyzed most predominantly by CYP2D2, followed by CYP3A2 and CYP3A1. In addition, the metabolism in RLM was remarkably inhibited by quinine (rat CYP2D inhibitor). On the contrary, S-CAR was metabolized most predominantly by CYP2D2, followed by CYP3A1/2 and CYP2C11. The metabolism in RLM was remarkably inhibited by quinine and ketoconazole (CYP3A2 inhibitor), followed by furafylline and α-naphthoflavone (CYP1A inhibitors). It has been reported that furafylline (25 μM) and ketoconazole (1 μM) could also inhibit CYP2C in rats, although furafylline and ketoconazole are selective inhibitors for human CYP1A and CYP3A, respectively (Eagling et al., 1998). It could be likely that ketoconazole (1 μM) but not furafylline (25 μM) inhibited CYP2C12, because furafylline weakly inhibit in rats compared with human by binding covalently to CYP apoprotein (Eagling et al., 1998). Imaoka et al. (1996) demonstrated in 7-week-old male rats that the major form was CYP2C11, followed by CYP3A2 (approximately 1/2 of CYP2C11) and CYP2D1 (approximately 1/3 of CYP2C11). As the S-CAR metabolic activity of CYP2C11 was about half of that of CYP2D2, the contribution of CYP2C11 against S-CAR metabolism would be greater than that against R-CAR, and therefore, might be indispensable. The combination of both the present results with recombinant CYPs and the reported content of each CYP in rat liver (Imaoka et al., 1996) suggest that CYP2D2 mainly catalyzed R-CAR metabolism, whereas CYP2D2 and CYP3A1/2 catalyzed S-CAR metabolism. Therefore, CYP2D subfamily was the predominant metabolizing enzyme class for R-CAR in both rats and humans. However, for S-CAR, the main contributing enzymes were CYP2D1/2 and CYP3A1/2 in rats and CYP1A2 in humans. It has been demonstrated that the species-specific isoforms of CYP1A, CYP2C, CYP2D, and CYP3A show appreciable interspecies differences in terms of catalytic activity, suggesting that some caution should be exercised while extrapolating...
Steroselective cytochrome P450 for carvedilol metabolism

metabolism data from animals to humans (Martignoni et al., 2006). A genetic polymorphism in CYP2D1 but not CYP2D2 affected metabolism and caused polymorphic variations in the pharmacokinetics profile of drugs (Hasegawa et al., 2014). There are possibly little effects of genetic polymorphism in CYP on CAR metabolism, because CYP2D2 mainly catalyze CAR metabolism in rats. These results to further investigate the involvement of other CYP isoforms such as CYP2C6 in CAR metabolism.

CYP2C11, which is a male-specific CYP isoform, is 50% of the total CYP content in adult male rat liver and is a homologue of the human CYP2C9 with 77% homology (Wójcikowski et al., 2013). CYP2C11 was largely involved in CAR metabolism compared with female-specific CYP2C12 (Fig. 2), suggesting that these differences could result in sex differences in CAR metabolism. It would be useful to further investigate the involvement of other CYP isoforms such as CYP2C6 in CAR metabolism.

Rats and dogs are used in the toxicity test of CAR (interview form for CAR Artist® tablets), http://www.info.pmda.go.jp/go/interview/2/430574_2149032F1021_2_A15_1F). However, there are some differences in contribution ratios of CYP isoforms in CAR metabolism. For example, similar contribution ratios of CYP2D1/2 in R-CAR and S-CAR metabolism were observed in rats, although CYP2D6 catalyzed R-CAR rather than S-CAR in human and the larger contribution ratios of CYP3A2 (Table 1). These differences could affect the development of CAR toxicity in rats and humans. It could be important to pay attention to species differences of CAR metabolism in toxicity test of CAR.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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