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Regular Article

Application of substrate depletion assay to evaluation of CYP isoforms responsible for stereoselective metabolism of carvedilol

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ABSTRACT

To evaluate the relative contribution of cytochrome P450 (CYP) isoforms responsible for carvedilol (CAR) oxidation, enantioselective metabolism of CAR was investigated in human liver microsomes (HLMs) and recombinant human CYPs by using the substrate depletion assay. CYP2D6 exhibited the highest contribution to the metabolism of *R*-CAR, followed by CYP3A4, CYP1A2, and CYP2C9, whereas the metabolism of the *S*-enantiomer was mainly mediated by CYP1A2, followed by CYP2D6 and CYP3A4. In HLMs, metabolism of *R*- and *S*-CAR was markedly inhibited by quinidine; *R*-CAR metabolism (57–61% decrease) was more inhibited than *S*-CAR metabolism (37–43% decrease), and furafylline and ketoconazole almost equally inhibited metabolism of both enantiomers by 25–32% and 30–50%, respectively. The absence of CYP2D6 in a mixture of five major recombinant CYP isoforms at the approximate ratio as in HLMs resulted in a 42% and 25% decrease in the metabolic activities for *R*- and *S*-CAR, respectively. Moreover, the absence of CYP1A2 in the mixture resulted in a 16% and 39% decrease in the metabolic activities for *R*- and *S*-CAR, respectively. Our results suggest the stereoselective metabolism of CAR is determined by not only the activity of CYP2D6 but also of CYP1A2 and CYP3A4.

1. Introduction

Carvedilol (CAR) is a third-generation β-adrenoceptor antagonist with not only β-blockage but also α_1-blockage (vasodilatation) [1]. This drug is used clinically as a racemic mixture of *R*- and *S*-CAR. CAR possesses both enantioselective pharmacodynamic and pharmacokinetic properties [2–5]. Although there is no difference in α-blocking activity between the enantiomers, the β-blocking activity of *S*-CAR is stronger than that of *R*-CAR [2,6]. The clearance of *R*-CAR is smaller than that of *S*-CAR in humans [3,4], and the metabolism of *R*-CAR is mainly mediated by polymorphic CYP2D6 [7], to form 4′- and 5′-hydroxy-CAR [8]. Although the clearance of *R*-CAR is further reduced in poor metabolizers (PMs) of CYP2D6 [4,5], considerable CAR metabolism was observed in the livers of PMs [3,5,8], suggesting that CAR is stereoselectively metabolized in humans, and other CYP isoforms contribute to this metabolism. In fact, Oldham and Clarke have reported that CYP2D6, CYP2C9, CYP2E1, CYP1A2, and CYP3A4 oxidize CAR to 4′-, 5′-, and 8-hydroxy, and *O*-desmethyl metabolites [8]. Because the two enantiomers of CAR differ in their pharmacological and pharmacokinetic properties, it is important to individually identify the CYP isoform(s) involved in their metabolism. However, a relative contribution of each CYP isoform to the overall metabolism of CAR is not fully known.

The identification of the CYP isoform(s) involved in the metabolism of a specific drug has generally been performed by measuring the production rate of metabolites involved in a specific metabolic pathway. However, because the metabolic processes of new drugs in the early stages of drug development and of toxic compounds are not completely elucidated, the disappearance rate of a compound from an incubation medium is useful for the identification of the responsible CYP isoform(s) [9–11]. Obach and Reed-Hagen reported a substrate depletion assay that provides a Michaelis constant (K_m) that is comparable to those from the conventional metabolite formation assay [12]. As this approach is applicable to older drugs for which metabolic profiles have not been reported, drugs for which metabolic pathways are multiple and complex, or drugs for which the metabolites are not

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commercially available [9], we have used this approach to reveal drug metabolizing enzymes for several psychotropic drugs including benzodiazepines such as clotiazepam, etizolam, and tofisopam [13]. We have also shown the usefulness of the substrate depletion assay to assess the linearity of pharmacokinetics of some β-blockers such as metoprolol, timolol, and propranolol [14]. Assessment of the human CYP isoforms involved in the biotransformation of a drug can be useful in defining the characteristics of its pharmacokinetic behavior, particularly if polymorphic or highly variably expressed enzymes are involved, and in the prediction of metabolic drug interactions. As mentioned above, CAR is metabolized to many oxidized metabolites as well as glucuronide by several CYP isoforms [8] and UDP-glucuronosyltransferase (UGT) isoforms [15]. However, there is little information available on the quantitative contribution of each CYP isoform to *R*- and *S*-CAR metabolism.

In the present study, we investigated the quantitative characterization of the human CYP isoforms responsible for the stereoselective biotransformation of CAR by measuring the disappearance rate of parent compounds. In addition, we propose a new method, which clarifies the effect of less active CYP isoforms like those found in polymorphic PMs, on the metabolic contribution of each CYP, in drug metabolic pathways involving multiple metabolic enzymes.

2. Materials and methods

2.1. Materials

Recombinant human cytochrome P450 enzymes (rhCYP1A2, rhCYP2C9, rhCYP2C19, rhCYP2D6, and rhCYP3A4) expressed in baculovirus-infected insect cells, insect cell control microsomes, pooled 150-donor human liver microsomes (HLMs), and HLMs from PMs of CYP2D6 (CYP2D6*4/*4 and CYP2D6*5/*5) were purchased from BD Gentest (Wobum, MA, USA). *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, quinidine, and sulfaphenazole 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). Monoclonal antibodies against human CYP1A2 (IH-MAB-1A2), CYP2D6 (IH-MAB-2D6), and CYP3A4 (IH-MAB-3A4) were obtained from BD Gentest. All other chemicals used were of the highest purity available.

2.2. Determination of CAR depletion rate

The incubation mixture (total volume, 0.2 mL) contained of 25 pmol/mL of each rhCYP isoform or 0.5 mg/mL of HLMs, 100 mM potassium phosphate buffer (pH 7.4), an NADPH-generating system (1.3 mM $NADP^+$, 3.3 mM G6P, 0.4 units/mL G6PDH, and 3.3 mM $MgCl_2$), and 0.3, 1, or 2 μM *R*- or *S*-CAR. Microsomal protein concentrations of all rhCYP were adjusted to 0.5 mg protein/mL by adding insect cell control microsomes. The reaction was initiated by the addition of the NADPH-generation system, following a 5-min pre-incubation at 37 °C. At appropriate time points, an aliquot of the reaction mixture was placed in a centrifuge tube containing two volumes of ice-cold acetonitrile including propranolol hydrochloride as an internal standard. After centrifugation at 10,000 × *g* for 3 min, the remaining concentration of CAR in the supernatant was determined.

rhCYP-based reconstructed HLMs were prepared by mixing major human CYP isoforms, which are involved in the metabolism of CAR, at almost the same ratio as in the HLMs described by Shimada et al. [16] (CYP1A2; 13%, CYP2C9; 20%, CYP2C19; 4%, CYP2D6; 2%, CYP3A4; 30%, 72% of total CYP). In addition, "reconstructed $HLM_{CYP2D6PM}$" and "reconstructed $HLM_{CYP1A2minus}$" were prepared by excluding rhCYP2D6 and rhCYP1A2 from the "reconstructed HLM", respectively. Metabolic incubation with the reconstructed HLMs (final concentration: 17.3 pmol/mL) was performed in the same manner as with each rhCYP isoform described above.

2.3. Effects of chemical inhibitors and anti-CYP antibodies

The effects of CYP-selective inhibitors and anti-CYP antibodies on the depletion of CAR enantiomers catalyzed by rhCYP isoforms or HLMs were evaluated. In inhibition assays, CAR enantiomers were used at the concentration of 1 μM. To confirm the relative contribution of CYP isoforms to the enantioselective metabolism of CAR, quinidine (a CYP2D6 inhibitor, at 1 and 10 μM), furafylline (a CYP1A2 inhibitor, at 2.5, 25, and 40 μM), α-naphthoflavone (a CYP1A2 inhibitor, at 1, 5, 20, and 50 μM), sulfaphenazole (a CYP2C9 inhibitor, at 1, 3, and 10 μM), and ketoconazole (a CYP3A4 inhibitor, at 0.1, 0.3, and 1 μM) were used. The inhibitory effects of anti-CYP antibodies on enantioselective CAR metabolism catalyzed by HLMs were examined by preincubating HLMs with the antibodies for 10 min on ice. Each monoclonal anti-CYP antibody was used at a concentration ranging from 10 to 100 μg/0.1 mg microsomal protein. The reaction was performed in a similar manner as that described above.

2.4. HPLC assay of CAR

R- and *S*-CAR concentrations were measured using a 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. with some modifications [17]. Briefly, chromatographic separation was achieved by using a Cosmosil $5C_{18}$-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). 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.

2.5. Estimation of the relative contribution of CYP isoforms to enantioselective CAR metabolism

The depletion rate constant (k_{dep}) was estimated from the logarithmic time course of the remaining CAR concentration in the reaction mixture by using linear regression, and then intrinsic clearance (CL_{int}) was calculated as a ratio of k_{dep}/CYP concentration in the reaction mixture:

$$CL_{int,\,CYP}(\mathrm{mL/min/nmol\ CYP}) = k_{dep} \Big/ \mathrm{CYP\ concentration}.$$

$CL_{int,\,CYP}$ estimated from the recombinant CYP system was extrapolated to HLMs (mg microsomal protein) based on total CYP concentration (0.32 nmol/mg microsomal protein) and expression percentage of the individual CYP enzyme in HLMs (CYP1A2, 13%; CYP2C9, 20%; CYP2C19, 4%; CYP2D6, 2%; CYP3A4, 30%) as follows:

$$CL'_{int,CYP}(\mathrm{mL/min/mg}) = CL_{int,\,CYP}(\mathrm{mL/min/nmol\ CYP}) \times 0.320\ \mathrm{nmol/mg}) \times \mathrm{expression\ \%\ for\ CYP\ enzyme}.$$