Chiral metabolism of propafenone in rat hepatic microsomes treated with two inducers

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Abstract

AIM: To study the influence of inducers of drug metabolism enzyme, β-naphthoflavone (BNF) and dexamethasone (DEX), on the stereoselective metabolism of propafenone in the rat hepatic microsomes.

METHODS: Phase I metabolism of propafenone was studied using the microsomes induced by BNF and DEX and the non-induced microsome was used as the control. The enzymatic kinetics parameters of propafenone enantiomers were calculated by regress analysis of Eadie-Hofstee Plots. Propafenone enantiomer concentrations were assayed by a chiral HPLC.

RESULTS: The metabolite of propafenone, N-desalkylpropafenone, was found after incubation of propafenone with the rat hepatic microsomes induced by BNF and DEX. In these two groups, the stereoselectivity favoring R(-) isomer was observed in metabolism at low substrate concentrations of racemic propafenone, but lost the stereoselectivity at high substrate concentrations. In the control group, no stereoselectivity was observed. The enzyme kinetic parameters were: (Km)ctrl group: R(-) 8.9±6, S (+) 94±4; BNF group: R(-) 105±6, S (+) 128±14; DEX group: R(-) 86±11, S (+) 118±16; (Vmax) ctrl group: R(-) 0.37±0.16, S (+) 0.72±0.07; BNF group: R(-) 0.37±0.15, S (+) 0.7±14; DEX group: R(-) 0.93±0.06, S (+) 1.04±0.09; (Clint) ctrl group: R(-) 8.9±1.1, S (+) 7.6±0; BNF group: R(-) 10.9±0.9, S (+) 8.9±0.7; DEX group: R(-) 10.9±0, S (+) 8.9±0.9. The enantiomeric differences in Km and Clink were both significant, but not in Vmax in BNF and DEX group. Whereas enantiomeric differences in three parameters were all insignificant in control group. Furthermore, Km and Vmax were both significantly less than those in BNF or DEX group. In the rat liver microsome inducted by BNF, nimodipine (NDP), decreased the stereoselectivity favoring R(-) isomer more than S (+)-isomer. The inhibition constant (Ki) of S (+)- and R(-)-propafenone, calculated from Dixon plots, was 15.4 and 8.6 mg·L⁻¹, respectively.

CONCLUSION: CYP1A subfamily (induced by BNF) and CYP3A4 (induced by DEX) have pronounced contribution to propafenone N-desalkylation which exhibited stereoselectivity depending on substrate concentration. The molecular base for this phenomenon is the stereo selectivity in affinity of substrate to the enzyme activity centers instead of at the catalyzing sites.

Subject headings propafenone/metabolism; mitochondria; liver; rat; optical rotation

INTRODUCTION

Propafenone, is a widely used antiarrhythmic agent administered as the racemic mixture of R(-) and S(+) enantiomers. The two enantiomers are equipotent in terms of sodium channel-blocking activity, but the main side effect, ie., β-adrenoreceptor-blocking action resides in the S (+)-isomer[1], and, therefore, information on stereoselective disposition of the racemate is of clinical relevance.

The main metabolic pathways of propafenone in vivo and in vitro involve CYP1A2 and CYP3A4 mediated N-desalkylation, CYP2D6 mediated 5-hydroxylation and UDPGT mediated glucuronidation[2-6]. N-desalkylpropafenone has the same electrophysiologic potency as 5-hydroxypropafenone and propafenone, and the plasma concentrations of N-desalkyl propafenone are similar to those of 5-hydroxypropafenone during chronic administration in human, therefore, N-desalkylpropafenone contributes to the antiarrhythmic effects of propafenone, especially in patients with poor metabolizer phenotype of CYP2D6[7-11], whether N-desalkylation exhibits stereoselectivity has not been addressed. Meanwhile, rat liver microsomes pretreated by specific inducers provide sound models to study metabolism in vitro[9-12], considering that β-naphthoflavone (BNF) is a typical inducer of CYP1A2 subfamily and dexamethasone (DEX) is a typical inducer of CYP3A4[17-21], this experiment studied the stereoselective metabolism of propafenone in rat hepatic microsomes induced by BNF and DEX.

Material AND METHODS

Chemicals and solutions

Dexamethasone (DEX), β-naphthoflavone (BNF), 7-ethoxyresorufin (ER), triacetyloleandomycin (TAO), NADPH, 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC), (R,S)-propafenone, R(-) and S(+) propafenone were supplied by Sigma Chemical Co (St. Louis, MO, US A). N-desalkylpropafenone was a generous gift from Prof. Tang YN (Xinhua Hospital, Shanghai). All other chemicals were obtained from the common commercial sources. Stock buffer (pH7.4): 1 mol·L⁻¹ pH7.4 Tris-HCl buffer 25 mL, 1 mol·L⁻¹ KCl 75 mL and 1 mol·L⁻¹ MgCl₂ 5 mL were mixed and diluted with water to 500 mL. NADPH solution: dissolve NADPH in ice-cold 10 g·L⁻¹ NaH CO₃.
solution to the desired concentration of 25 mmol·L⁻¹. The solution should be freshly prepared just before the incubation.

**Preparation of hepatic microsomes**

Sprague-Dawley rats (male, 170-210 g) were divided into three groups. One group received three daily intraperitoneal injection of 80 mg·kg⁻¹ BNF (dissolved in oil); the second group received three daily DEX (132 mg·kg⁻¹·d⁻¹, ig) and the third group was used as the non-treated control. About 24 h after the last treatment and with no food supplied for 16 h before taking the livers, the rats were sacrificed by decapitation. Liver samples were excised and perfused by the ice-cold physiological saline to remove blood and homogenized in ice-cold Tris buffer. Hepatic microsomes were prepared with the ultracentrifugation methods[22,23]. All manipulations were carried out in cold bath. Pellets were re-suspended in sucrose-Tris buffer (pH 7.4)(95:5, mass to volume ratio) and immediately stored at -30°C. Protein and cytochrome P450 contents were estimated according to the methods of Zeng et al[24] and Omura et al[25], respectively. Enzymatic activity of CYP1A was measured according to the method of Klotz et al[26], and expressed as initial velocity of O-deethylation of 7-ethoxyresorufin (activity of EROD). Enzymatic activity of CYP3A4 was determined according to the method of Wrighton et al[27], and expressed as the extent of P450-MI complex (absorbance difference per gram of protein between 456 nm and 510 nm) using triacetyloleandomycin as substrate. Incubation of propafenone with rat hepatic microsomes The incubation mixture contained microsomal protein (1.6 g·L⁻¹), stock buffer (pH 7.4) bubbled with oxygen for 1 min and racemic propafenone as substrate. After 5 min preincubation, reaction was started by adding 10 µL NADPH solution. The final volume was 250 µL. For kinetic experiments, racemic propafenone was used at concentrations of 10, 20, 40, 80, 160, and 320 mg·L⁻¹ and the incubation time was 30 min. For the time-dependent experiments, the substrate concentration used was 10 mg·L⁻¹. For inhibition experiments, nimodipine was used as inhibitors (at 0, 8, 16, 32 mg·L⁻¹) and incubated simultaneously with racemic propafenone (50, 100 mg·L⁻¹). After the indicated time, the reaction was terminated by adding 750 µL chlorform. The mixture was vortexed for 3 min, then centrifuged at 2 000 g for 10 min. The organic layer was transferred to a clean tube and evaporated to dryness under a gentle stream of air.

GITC solution (in acetonitrile) and methanol containing 14 g·L⁻¹ triethamine were added and the tube was capped and allowed to react for 30 min at 35°C. After evaporation of organic solvents, the residues were reconstituted with 100 µL methanol, and 20 µL was injected into HPLC system.

**HPLC procedure for determining propafenone enantiomer in the rat hepatic incubates**

Enantiomers of propafenone were quantitated with an HPLC system with UV detection (λ = 254 nm)[28]. A 5-μm reverse phase column (Shimpack CLC- ODS 15 cm×4.6 mm) was used with a flow rate of 0.8 mL/min. The mobile phase was a mixture of methanol -water-glacial acetic acid (67:33:0.05).

**Statistical analysis**

The maximum velocity (υₘᵢₓ) and Michaelis-Menten constant (Kₘ) values for propafenone enantiomer were determined by regression analysis of Edie-Hofstee plots. The FAs of three determinations of υₘᵢₓ and Kₘ was calculated for each substrate and metabolic reaction. Intrinsic clearance was calculated by the ratio of υₘᵢₓ/Kₘ. All statistical difference was tested by unpaired t test.

**RESULTS**

A baseline separation between the diastereomers of S(+) and R(-)- propafenone was achieved, with the retention time being 23 min and 28 min, respectively. The HPLC system also allowed monitoring the formation of N-desalkylpropafenone. The retention time was 8 min and 10 min for diastereomer of N-desalkylpropafenone, respectively. The amount of diastereomers of N-desalkylpropafenone were increasing while those of propafenone were decreasing during 30 min incubation with the rat hepatic microsomes induced by DEX and BNF. Typical chromatograms were showed in Figure 1. Quantitation was performed by external standardization. Calibration curves were linear at a range of 0.5 to 320 mg·L⁻¹ for each enantiomer of propapfenone. The LOQ was 0.5 mg·L⁻¹ (S/N = 10, n = 5) for each enantiomer. The inter-assay and intra-assay variability averaged 8.5% for both enantiomers. The method recovery averaged 77.1% for both enantiomers.

**Figure 1** Chiral high performance liquid chromatogram of racemic propafenone in rat liver microsomal incubates after 30 min incubation. A:BNF pre-treated B:without incubation.

Peaks 1,2: Diastereomers of S(+)–propafenone and R(-)-propafenone; Peaks 3,4: Dia stereomers of metabolite (N-desalkylpropafenone)
Induction of rat hepatic metabolizing enzymes

In DEX group, the extent of P450-MI complex (an indicator of activity of CYP3A4) was significantly more than the control or BNF group (P<0.001, Table 1). In BNF group, the initial velocity of deethylation of 7-ethoxyresorufin (an indicator of activity of CYP1A) was significantly more than in the control or DEX group (about 20-fold, P<0.001). Therefore, CYP1A subfamily was successfully induced by BNF and CYP3A4 by DEX, which provided sound enzymatic sources for getting information on CYP1A and CYP3A4 mediated N-desalkylation of propafenone.

Table 1 The amount and activity of P450 in rat liver microsomes (x±s, n = 3)

| Pretreat | P450 in pro /μmol·g⁻¹ | Extent of P450-MI complex, A | Activity of EROD /μmol·min⁻¹·µg⁻¹ |
|----------|------------------------|-----------------------------|----------------------------------|
| Control  | 0.95±0.15              | 0.9±0.2                     | 0.22±0.04                        |
| BNF      | 1.42±0.21              | 2.2±0.4                     | 3.87±0.20                        |
| Dex      | 1.11±0.17              | 18.3±3.6                    | 0.14±0.02                        |

αP<0.01, vs BNF or control; βP<0.01, vs Dex or control.

Impact of substrate concentration on stereoselective metabolism of propafenone

At 10 mg·L⁻¹ concentration of racemic propafenone, stereoselectivity was observed in DEX and BNF group, but not in control group (Table 2). The depletion of R(-)-isomer was faster than that of S(+)-isomer.

Table 2 Ratio of S(+)/R(-) propafenone at different concentrations in rat liver microsomal incubates (x±s, n = 3)

| Enantiomer | Pretreat |
|------------|----------|
| Concentration / mg·L⁻¹ | Control | Dex | BNF |
| 5           | 1.01±0.016 | 1.17±0.062 | 1.10±0.019 | 1.04±0.19 |
| 10          | 1.02±0.012 | 1.10±0.057 | 1.06±0.015 |
| 20          | 0.99±0.016 | 1.08±0.018 | 1.03±0.002 |
| 40          | 0.97±0.026 | 1.05±0.030 | 1.04±0.000 |
| 80          | 0.97±0.024 | 1.01±0.017 | 1.02±0.005 |
| 160         | 0.98±0.012 | 1.03±0.019 | 1.00±0.005 |

αP<0.01, vs control; βP<0.01, vs 160 mg·L⁻¹.

Concentration-time curves and ratio of S(+)/R(-) propafenone concentration

The ratio of S/R was in unity in control group from the incubation time of 0 to 30 min, whereas in DEX or BNF group, the ratio of S/R increased and was significantly different with the corresponding ratio in control group at 8 and 30 min (P<0.01, 0.05, Table 3). Moreover, the ratio of S/R in DEX group at incubation time of 30 min was significantly higher than that in BNF.

Table 3 Ratio of S(+)/R(-) propafenone concentration in rat liver microsomal incubates (x±s, n = 3)

| Group  | 0      | 3      | 8      | 20     | 30 (min)       |
|--------|--------|--------|--------|--------|----------------|
| Control| 1.000  | 1.017±0.010 | 0.997±0.016 | 1.006±0.012 | 1.016±0.016 |
| Dex    | 1.000  | 1.007±0.003 | 1.044±0.011 | 1.076±0.019 | 1.170±0.050 |
| BNF    | 1.000  | 1.005±0.002 | 1.031±0.012 | 1.068±0.023 | 1.094±0.017 |

αP<0.01, vs 8 min; βP<0.05, vs BNF group; γP<0.01, δP<0.05, ϵ vs control.

Enzymatic kinetic parameters for propafenone metabolism in hepatic microsomes

Depletion of propafenone could be described by Michaelic-Menten kinetics. Km had no statistical difference between the two enantiomers in control microsomes, whereas the enantiomeric difference in Km was significant in the microsomes induced with DEX or BNF (S>R, P<0.05, Table 4). There was significant difference for Clint between the two enantiomers (S<R, P<0.05, Table 4) in DEX or BNF group.
but not in control group. The $K_m$ of S(+)-isomer in DEX, or S(+)- or R(-)-isomer in BNF group was significantly higher than the corresponding enantiomer in control group ($P<0.05, 0.01$, Table 4). The $V_{max}$ of S(+)-isomer in DEX group, or S(+)- or R(-)-isomer in BNF group, was significantly higher than the corresponding enantiomers in the control group ($P<0.05, 0.01$, Table 4). Difference for Clint between the two enantiomers in DEX or BNF group and the corresponding enantiomer in control group was insignificant. Moreover, the $K_m$ of R(-)-propafenone in DEX group was significantly lower than that in BNF group ($P<0.05$, Table 4).

### Table 5 The stereoselective effects of nimodipine on metabolic depletion of propafenone ($x±s, n=3$)

| Group               | Nimodipine $/mg·L^{-1}$ | S(+)-propafenone $/mg·L^{-1}$ | R(-)-propafenone $/mg·L^{-1}$ | S/R       |
|---------------------|--------------------------|-------------------------------|-------------------------------|-----------|
| DEX                 | 0                        | 2.10±0.04                    | 1.75±0.14                    | 1.20      |
| DEX                 | 8                        | 2.32±0.26                    | 2.10±0.21                    | 1.1       |
| 0DEX                | 16                       | 3.81±0.11                   | 3.62±0.13                    | 1.0       |
| 6DEX                | 32                       | 4.30±0.13                   | 4.17±0.26                    | 1.03      |

$\Delta P<0.01$, vs S(+)-propafenone in DEX without nimodipine; $\triangle P<0.05$, $\Delta P<0.01$, vs the corresponding enantiomer in control DEX or BNF group.

### DISCUSSION

Due to the capabilities of highly efficient separation and sensitive determination of enantiomers in microsome incubates, chiral chromatography is extremely valuable to study stereoselectivity of racemate metabolism[29-34]. So far as we are aware, we took the lead in gathering the information on stereoc hemistry of propafenone metabolism by chiral HPLC method.

Previously, we observed that the glucuronidation of propanolol in rat hepatic microsome has stereoselectivity of S(-)-propranolol, and that the induction of phenobarbital reduced this stereoselectivity[35]. The phase I metabolic stereoselectivity of propranolol was reversed by the induction of BNF and increased by the induction of phenobarbital[36]. Phenobarbital instead of BNF induced the stereoselective difference of $C_{min}$ in glucuronidation of ofloxacin[37]. However, the induction of DEX or BNF in this study vested propafenone metabolism with stereoselectivity in rat hepatic microsomes. It is thus clear that different inducers may have different impacts on some racemate metabolism.

The enantiomers of a racemic drug may differ in metabolic behavior as a consequence of stereoselective interaction with hepatic microsomes[38-42]. The underlying mechanism of stereoselectivity in metabolism, as many studies have shown, was enantiomeric difference in $V_{max}$ (an index of enzymatic catalyzing ability) and/or in $K_m$ (an index of enzyme affinity to the substrate). For example, the stereoselective N-demethylation of chlorpheniramine was due to enantiomeric differences in $K_m$[43]. Whereas there were little or no difference in $K_m$ of the enantiomers of ofloxacin, the stereoselectivities in glucuronidation were caused by enantiomeric differences in $V_{max}$[44]. The $V_{max}$ of the O-demethylation of (-)-tramadol was 1.6 times that of (+)-isomer, but the Km for both enantiomers was same, thus resulted in its stereos elective O-demethylation[45]. Recently, we have also proved that stereoselectivity of propranolol cytochrome P450 metabolism in the rat hepatic microsomes was due to the stereoselectivity of the catalyzing function in enzyme[46]. In this in vitro study, stereoselectivity of propafenone occurred in $K_m$ and Clint in the rat hepatic microsomes induced by DEX or BNF, but not in $V_{max}$.[47]. Combining with the interesting results of Table 2 that stereoselectivity depends on substrate concetration, we suppose that stereoselectivity at low substrate concentration was mainly due to the enantiomeric difference of the enzyme affinity to the substrate, and that insignificant enantiomeric difference in catalyzing abilities resulted in the abolished stereoselectivity at high substrate concentration. Fujita et al[46] also reported that stereoselectivity of propranolol in rat liver microsomes was sometimes altered when the substrate concentration was varied. Augustijns et al[48] observed that the enantiomeric ratio (R/S) of desethylchloroquine was dependent on concentration, and ranged from 8 at 1 microM to 1 at 300 microM. Mutual enantiomer-enantiomer interaction on studies at low concentration (1-5 microM) revealed that the formation of (R)-desethylchloroquine was strongly inhibited by (S)-chloroquine. In this in vitro metabolism,
enantiomer-enantiomer interaction at enzyme activity centers may also exist at low concentration, resulting in enantiomeric difference of the enzyme affinity to the substrate. This needs to be addressed by additional experiments.

Table 3 indicated that the stereoselectivity in DEX was stronger than in BNF. It may be explained by the difference in $K_{m}$ of R(-)-propafenone between DEX and BNF group and that the affinity of R(-)-PPF with CYP3A4 was higher than that with CYP1A1, and that of S(+)-PPF with CYP3A4 was similar with CYP1A. Table 1 showed that CYP1A1 and CYP3A4 were significantly induced by BNF and DEX, respectively, and this agreed with the well-known documents. In BNF or DEX group, the $v_{max}$ was also significantly higher than that in the control group (about 1.5-fold), which indicated that CY P1A1 and CYP3A4 contributed to the metabolism of propafenone. This substantiated the methods used by Bottsch et al. In their study, CYP1A2 and CYP3A4 were identified in N-desalkylation using specific antibodies and inhibitors and stably expressed cytochrome P450. $K_{m}$ in the control group was significantly lower than that in DEX or BNF group, which indicated that other enzyme with high affinity to substrate involved in metabolism of propafenone. CYP2D6 which had very low value of $K_{m}$ might be one of such enzymes. Due to the lower value of both $K_{m}$ and $v_{max}$ in control group, the Cient of propafenone enantiomer was not different from that in DEX or BNF group.

The competitive inhibition model (propafenone/nimodipine) suggested that propafenone and nimodipine were both substrates of the same coenzyme. Because nimodipine was as paccific substrate of CYP3A4[4,41], the results of inhibition experiment also proved that CYP3A4 contributed to propafenone metabolism. Drug interaction of enantiomer with specific inhibitor of P450 is an important tool in in vivo metabolism. In their study, Fontaine et al. concluded that CYP2D6-mediated methadone metabolism exhibited stereoselectivity. The fact that the AUC ratio for the two enantiomers of reboxetine was minimally affected by ketoconazole treatment substantiated the methods used by Bottsch et al. In their study, CYP1A2 and CYP3A4 were identified in N-desalkylation using specific antibodies and inhibitors and stably expressed cytochrome P450. $K_{m}$ in the control group was significantly lower than that in DEX or BNF group, which indicated that other enzyme with high affinity to substrate involved in metabolism of propafenone. CYP2D6 which had very low value of $K_{m}$ might be one of such enzymes. Due to the lower value of both $K_{m}$ and $v_{max}$ in control group, the Cient of propafenone enantiomer was not different from that in DEX or BNF group.

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