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Article

Conversion and pharmacokinetics profiles of a novel pro-drug of 3-*n*-butylphthalide, potassium 2-(1-hydroxypentyl)-benzoate, in rats and dogs

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Abstract

Potassium 2-(1-hydroxypentyl)-benzoate (*dl*-PHPB) is a novel pro-drug of 3-*n*-butylphthalide (*dl*-NBP) that is used to treat ischemic stroke. Currently, *dl*-PHPB is in phase II–III clinical trials in China. In this study, we investigated the conversion and pharmacokinetics profiles of dl-PHPB *in vitro* and *in vivo*. The conversion of *dl*-PHPB to *dl*-NBP was pH- and calcium-dependent, and paraoxonase was identified as a major enzyme for the conversion in rat plasma. The pharmacokinetics, tissue distribution and excretion of *dl*-PHPB were studied and compared with equal-molar doses of *dl*-NBP in rats and dogs. The *in vivo* studies showed that *dl*-PHPB could be quickly and completely converted to *dl*-NBP. The plasma concentration-time course of converted dl-NBP after intravenous *dl*-PHPB administration was nearly the same as that after equal-molar dl-NBP. The C_{max} and AUC of *dl*-NBP after oral *dl*-PHPB administration in rats and dogs were higher by 60% and 170%, respectively, than those after oral *dl*-NBP administration. Analysis of the tissue distribution of *dl*-PHPB revealed that converted *dl*-NBP was primarily distributed in fat, the brain and the stomach. In the brain, the levels of *dl*-NBP were relatively higher after *dl*-PHPB treatment by orally than after treatment with equal-molar *dl*-NBP. Approximately 3%–4% of *dl*-NBP was excreted within 72 h after dosing with *dl*-PHPB or *dl*-NBP, but no *dl*-PHPB was detected in urine or feces excrements. Our results demonstrate that the conversion of *dl*-PHPB is fast after oral or intravenous administration. Furthermore, the bioavailability of *dl*-PHPB was obviously better than that of *dl*-NBP.

Keywords: potassium 2-(1-hydroxypentyl)-benzoate (*dl*-PHPB); pro-drug; 3-*n*-butylphthalide (*dl*-NBP); ischemic stroke; pharmacokinetics; bioavailability

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Introduction

Potassium 2-(1-hydroxypentyl)-benzoate (*dl*-PHPB), a potential pro-drug of 3-*n*-butylphthalide (*dl*-NBP) with a novel molecular structure, has been developed as an anti-cerebral ischemia agent with extremely high solubility in water[1]. The phase I clinical trial of *dl*-PHPB has been completed, and *dl*-PHPB is now in phase II-III clinical studies.

Dl-NBP is an oily compound with a boiling point of 140 –141 °C (320 Pa) that was originally isolated from the seeds of *Apium graveolens* Linn, or Chinese celery[2]. It has been widely used for ischemic stroke and has shown good therapeutic effects in China[3-7]. However, *dl*-NBP is hydrophobic and is difficult to be intravenously administered, which limits the use of *dl*-NBP in patients with cerebral ischemic stroke[7]. Additionally, the bioavailability of *dl*-NBP is very low[7]. *Dl*-PHPB has been designed and synthesized by the Department of Medicinal Chemistry, Institute of Materia Medica, Chinese Academy of Medical Sciences as a pro-drug of *dl*-NBP with very good solubility in water. *Dl*-PHPB can be converted to an active metabolite (*dl*-NBP) under certain chemical (*ie*, acidic) and biological conditions. Pharmacological studies have demonstrated that *dl*-PHPB exhibited similar or stronger neuroprotective effects than *dl*-NBP at equal-molar doses[1, 8-10]. The mechanisms underlying the anti-stroke properties of *dl*-PHPB include increased regional cerebral blood flow in ischemic zones and inhibited platelet aggregation[1, 8, 9]. Recent studies have shown that *dl*-PHPB improved cognitive defects by attenuating amyloid and tau protein pathologies in APP/PS1 transgenic mice, promoting long-term potentiation (LTP) in $A\beta_{1-42}$-injected rats and APP/PS1 mice[10-12]. Additionally, *dl*-PHPB protected neurons against H_2O_2-induced apoptosis in human neuroblastoma SK-N-SH cells by modulating

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apoptosis-related proteins; the PKC signaling pathway may also be involved in these mechanisms[13].

Although the pharmacological properties of *dl*-PHPB have been intensively investigated, its conversion, absorption, distribution, metabolism and excretion have not been well understood. Only a few studies have investigated the conversion of *dl*-PHPB in rat plasma[1]. Thus, the elucidation of the conversion and metabolism of *dl*-PHPB for clinical pharmacology and safety evaluations is very important.

In this study, we assessed *in vitro* studies to reveal the conversion mechanisms of *dl*-PHPB to *dl*-NBP and considered non-enzymatic acidic and alkaline solutions, calcium-dependent effect and plasma enzyme-mediated conversions. We also carried out *in vivo* studies to compare the pharmacokinetics, distribution and excretion of *dl*-PHPB with its metabolite *dl*-NBP after oral and intravenous administration in SD rats and Beagle dogs.

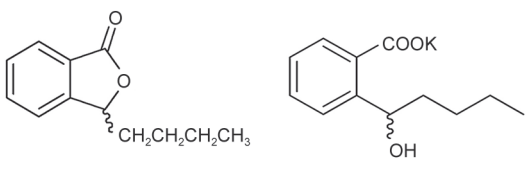
Materials and methods

Animals

SD rats (male and female, 190–210 g) were purchased from Vital River Lab Animal Technology Co, Ltd (Beijing, China), and Beagle dogs were obtained from Marshall Biotechnology Co, Ltd (Beijing, China). Animals were allowed free access to food and water in a temperature-controlled environment at 22 °C–25 °C during the experimental period. The experiments were performed in accordance with the guidelines for the care and use of laboratory animals and were approved by the Animal Care Committee of the Peking Union Medical College and the Chinese Academy of Medical Sciences (Beijing, China).

Chemicals and materials

Dl-PHPB and *dl*-NBP were offered by the Department of Synthetic Pharmaceutical Chemistry of the Institute of Materia Medica (Beijing, China) with purities of 99.1%, and 99.0%, respectively. The chemical structures of the two compounds are shown in Figure 1. Internal standards of 4-biphenylacetic acid and diazepam were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP, Beijing, China). Sodium *bis*-*p*-nitrophenyl phosphate (BNPP), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), phenylmethylsulfonyl fluoride (PMSF), teicoplanin, donepezil, ethylenediamine tetraacetic acid disodium salt dehydrate (EDTA-Na_2), ethyleneglycol-*bis* (2-aminoethylether)-tetraacetic acid (EGTA), and sodium fluoride (NaF) were purchased from Sigma-Aldrich (St Louis, MO, USA). High-performance liquid chromatography (HPLC) grade methanol was purchased from Burdick & Jackson Company (Muskegon, MI, USA). All other reagents were of analytical grade. Deionized water used throughout the study was purified using a Millipore water purification system (Milford, MA, USA).



dl-NBP *dl*-PHPB

Figure 1. Structures of *dl*-NBP and *dl*-PHPB.

Fresh whole blood was collected and pooled from healthy SD rats and beagle dogs with heparin or EDTA-Na_2 as an anticoagulant. Plasma was separated by centrifugation at 1123×*g* at 4 °C for 10 min. All pooled plasma was stored at -70 °C and thawed at 4 °C before assays.

Conversion of *dl*-PHPB in solutions of different pH values

Appropriate amounts of deionized water, 1.0 mol/L HCl, and 1.0 mol/L NaOH were used to make solutions with 1.0, 3.0, 7.0, and 9.0 pH. Reactions were initiated by adding an aliquot of a stock solution of *dl*-PHPB (1 mg/mL, in methanol) to obtain a final concentration of 10 μg/mL. The samples were allowed to settle for various time intervals from 0 to 360 min at 37 °C. An internal standard at a final concentration of 5 μg/mL was added to each sample, and the samples were analyzed without any pre-treatment by HPLC as described below. A control sample of *dl*-PHPB (10 μg/mL in methanol) or *dl*-NBP (7.8 μg/mL in methanol) was prepared and analyzed. For each sample, three replicated operations were performed. Residual percentage versus pH value was plotted, and degradation trends were observed.

Residual% of *dl*-PHPB or *dl*-NBP was calculated as follows:

$$\text{Residual\%} = A/B\ (\text{control}) \times 100\%$$

where A represents the concentration of *dl*-PHPB or *dl*-NBP produced by the solutions with different pH values and various time intervals, and B represents the control concentration of *dl*-PHPB or *dl*-NBP.

Conversion of *dl*-PHPB in SD rat plasma

SD rat plasma with heparin as an anticoagulant was pre-incubated at 37 °C for 5 min. The reaction was initiated by adding *dl*-PHPB solution (1 mg/mL, in methanol) to achieve a final concentration of 10 μg/mL. The temperature was maintained at 37 °C, representing body temperature, for the treatment of the plasma samples. A control sample of *dl*-PHPB (10 μg/mL in methanol) or of *dl*-NBP (7.8 μg/mL in methanol) was prepared. At different time intervals (0 to 360 min) at 37 °C, a sample of 200 μL was withdrawn. The reaction was stopped by adding 50 μL of saturated EDTA-Na_2 solution (0.3 mol/L) and by placing the samples on ice. For each sample, three replicated operations were performed. Residual percentage versus time was plotted. The quantifications of *dl*-PHPB and its active metabolite (*dl*-NBP) in these samples were performed by HPLC methods as described below.

Residual % of *dl*-PHPB or *dl*-NBP was calculated as follows:

$$\text{Residual\%} = A/B\ (\text{control}) \times 100\%$$

where A represents the concentration of *dl*-PHPB or *dl*-NBP

produced by the incubation in rat plasma with different time intervals, and B represents the control concentration of *dl*-PHPB or *dl*-NBP.

Effects of esterase inhibitors on *dl*-PHPB conversion

To characterize the esterases responsible for *dl*-PHPB conversion, *dl*-PHPB was incubated in rat plasma with heparin as an anticoagulant in the presence of various esterase inhibitors. This assay was conducted according to described methods, with modifications[14-17]. Briefly, the rat plasma (100 μL) was mixed with 350 μL of 50 mmol/L Tris-HCl buffer (pH 7.4) and either 50 μL of the inhibitor solution (in 10% *v/v* DMSO in water) or 50 μL of 10% DMSO (as control). The reaction was initiated by adding 20 μL of *dl*-PHPB solution (300 μg/mL, in methanol). The mixtures were incubated at 37 °C for 2 h. The reaction was stopped using 80 μL of saturated EDTA-Na_2 solution, and the tubes were placed on ice.

The quantification of *dl*-PHPB and *dl*-NBP in these samples was performed by HPLC methods as described below. The conversion of *dl*-PHPB was calculated as follows:

Conversion of *dl*-PHPB%=A/B (control)×100%

where A and B (control) represent the *dl*-NBP amounts produced by the incubation with and without the inhibitor, respectively.

Conversion of *dl*-PHPB in SD rats

The *vivo* conversion of *dl*-PHPB was studied in SD rats with intravenous injections of 20 mg/kg *dl*-PHPB. The plasma concentration time curves of *dl*-PHPB and its metabolite *dl*-NBP were measured by HPLC methods.

Briefly, 300 μL blood samples from the femoral vein were collected into tubes containing 10 μL of saturated EDTA-Na_2 at different time intervals after dosing. The blood samples were immediately centrifuged at 1123×*g* for 5 min, and the plasma was stored at -70 °C until HPLC analysis.

Plasma pharmacokinetics

Comparative studies on plasma pharmacokinetics of *dl*-PHPB and *dl*-NBP were carried out in Beagle dogs and SD rats. According to previous reports[1], the effective doses of *dl*-PHPB in rats subjected to acute focal cerebral ischemia-reperfusion were 5–10 mg/kg and 50–100 mg/kg by intravenous or oral administration, respectively. Thus, the following dosage ranges were selected to cover the effective dose of *dl*-PHPB. The concentration time curves of *dl*-PHPB and its active metabolite (*dl*-NBP) were measured after treatment with *dl*-PHPB intravenously (5, 10, and 20 mg/kg in SD rats or 2, 4, and 8 mg/kg in Beagle dogs) and orally (50, 100, and 200 mg/kg in SD rats or 10, 30, and 100 mg/kg in Beagle dogs). According to the molecular weights of the two compounds, *dl*-NBP was given at 15.6 mg/kg in SD rats and 6.2 mg/kg in Beagle dogs intravenously and at 156.0 mg/kg in SD rats and 23.4 mg/kg in Beagle dogs orally. Then, 300 μL blood samples were collected at different time intervals after dosing, according to the procedure described above. The plasma was stored at -70 °C until HPLC analysis.

Tissue distribution and excretion

SD rats were given *dl*-PHPB (10 mg/kg intravenously or 100 mg/kg orally) or equimolar doses of *dl*-NBP (7.8 mg/kg intravenously or 78 mg/kg orally). Tissues from the heart, liver, spleen, lung, kidney, stomach, small intestine, fat, and brain were obtained at 20, 60, 120, and 240 min by oral administration and at 20, 60, and 240 min by intravenous administration, respectively. Urine and feces samples were collected pre-dose and at 0–4, 4–8, 8–12, 12–24, and 24–36 h or at 24–48, 36–48, and 48–72 h post-dose. The total urine volume and total feces weight were recorded after each sample collection. The tissue, urine and feces samples were stored at -70 °C until analysis.

Quantitative analysis of dl-PHPB and dl-NBP

Preparation of plasma and urine samples For every 100 μL aliquot of pooled plasma (or urine) samples, 300 μL of methanol containing 10 μg/mL of internal standard were added. Mixtures were mixed for 1 min and centrifuged for 10 min at 12 000×*g*. A 200 μL aliquot of the supernatant was taken and filtered through a 0.22-μm membrane. A 60 μL aliquot was used for HPLC analysis.

Preparation of tissues and feces samples Each feces sample was thawed and placed in 5 parts (1 g: 5 mL) methanol. The mixture was homogenized by a motor-driven homogenizer (Fisher Scientific, Pittsburgh, PA, USA) and subjected to ultrasound treatment for 15 min. A 5 mL aliquot of the mixture was removed and centrifuged at 3500×*g* for 10 min. A 400 μL aliquot of the supernatant was taken and filtered through a 0.22-μm membrane. A 60 μL aliquot was used for HPLC analysis.

HPLC analysis

The concentrations of *dl*-PHPB and *dl*-NBP in plasma were determined by previously described HPLC methods[18-20]. An Agilent 1100 (Palo Alto, CA, USA) instrument was used. Standard curves with concentrations ranging from 0.05 to 60 μg/mL *dl*-PHPB and 0.01 to 60 μg/mL *dl*-NBP in plasma and urine exhibited good linearity with correlation coefficients >0.998. The limits of the *dl*-PHPB and *dl*-NBP assays were 0.05 μg/mL and 0.01 μg/mL, respectively, with acceptable precision and accuracy. The assay precision (deviation) was <15%, whereas the assay accuracy was 85%–115%. The mean absolute percentage recoveries of *dl*-PHPB and *dl*-NBP were more than 70% from spiked plasma, urine, tissues, and feces samples determined at different concentrations of quality control (QC) samples. QC samples with three concentration levels were prepared in control stabilized samples at final concentrations of 0.5, 10, and 50 μg/mL for *dl*-PHPB and 0.1, 10, and 50 μg/mL for *dl*-NBP. Intra- and inter-day plasma assay variability were less than 10%. HPLC methods for the determinations of *dl*-PHPB or *dl*-NBP were reliable and reproducible because the % CV and % bias were below 15% for all theoretical con-

centrations of *dl*-PHPB and *dl*-NBP.

Data analysis

First order rate constants (K) of conversion were calculated from the slopes of linear plots of residue percentage against time, and the corresponding half-life was obtained from the equation: $t_{1/2}$=0.693/K. All values were obtained from three independent determinations, and the mean values of the constants were calculated. The pharmacokinetic parameters were analyzed using 3P97 computer software package (Chinese Pharmacology Society, Beijing, China). The statistical analysis of outcome parameters was performed using SPSS version 13.5 (SPSS Inc, Chicago, IL, USA). The statistical evaluation was performed using analysis of variance (ANOVA) followed by multiple comparison tests by Duncan's method. The level of significance was set at P<0.05.

Results

Conversion of *dl*-PHPB to *dl*-NBP *in vitro* and *in vivo*

The *in vitro* and *in vivo* conversions of *dl*-PHPB to *dl*-NBP are shown in Figure 2. To study the *in vitro* conversion, *dl*-PHPB was added to distilled water of different pH values (1.0, 3.0, 7.0, and 9.0) or rat plasma and incubated at 37 °C for different periods. The *in vitro* conversion of *dl*-PHPB into *dl*-NBP showed a significant pH dependency. The level of *dl*-PHPB decreased rapidly and was undetectable at pH 1.0 after 30 min of incubation, whereas the level of *dl*-NBP increased. However, *dl*-PHPB was stable in neutral and alkaline solutions. The residue concentrations of *dl*-PHPB were 93% and 96% at initial pH levels of 7.0 and 9.0 after 4 h of incubation, respectively. At the same time, lower levels of *dl*-NBP (approximately 7% and 5% of the maximum) were detected in the solutions (Figures 2A, 2B). The conversion rates agreed with the first order kinetics shown in Table 1. The conversion in rat plasma was even more rapid. After 20 min of incubation in rat plasma at 37 °C, more than 90% of *dl*-PHPB was converted into *dl*-NBP (Figure 2C).

The *in vivo* conversion studies were carried out in SD rats. *Dl*-PHPB was intravenously injected at 20 mg/kg body weight. The mean plasma concentration time curves of *dl*-PHPB and its active metabolite are shown in Figure 2D. After injecting *dl*-PHPB, the conversion of *dl*-PHPB to *dl*-NBP was very rapid in the rats and could not be detected after 30 min; *dl*-NBP appeared at high levels immediately after injection. The concentrations of the active metabolite *dl*-NBP were at least 3-fold higher than those of *dl*-PHPB at the same time points. The pharmacokinetic parameters of the active metabolite *dl*-NBP are listed in Table 2. A two-compartment open model provided the best fit to the plasma concentration time profiles of *dl*-NBP obtained in rats after intravenous treatment with

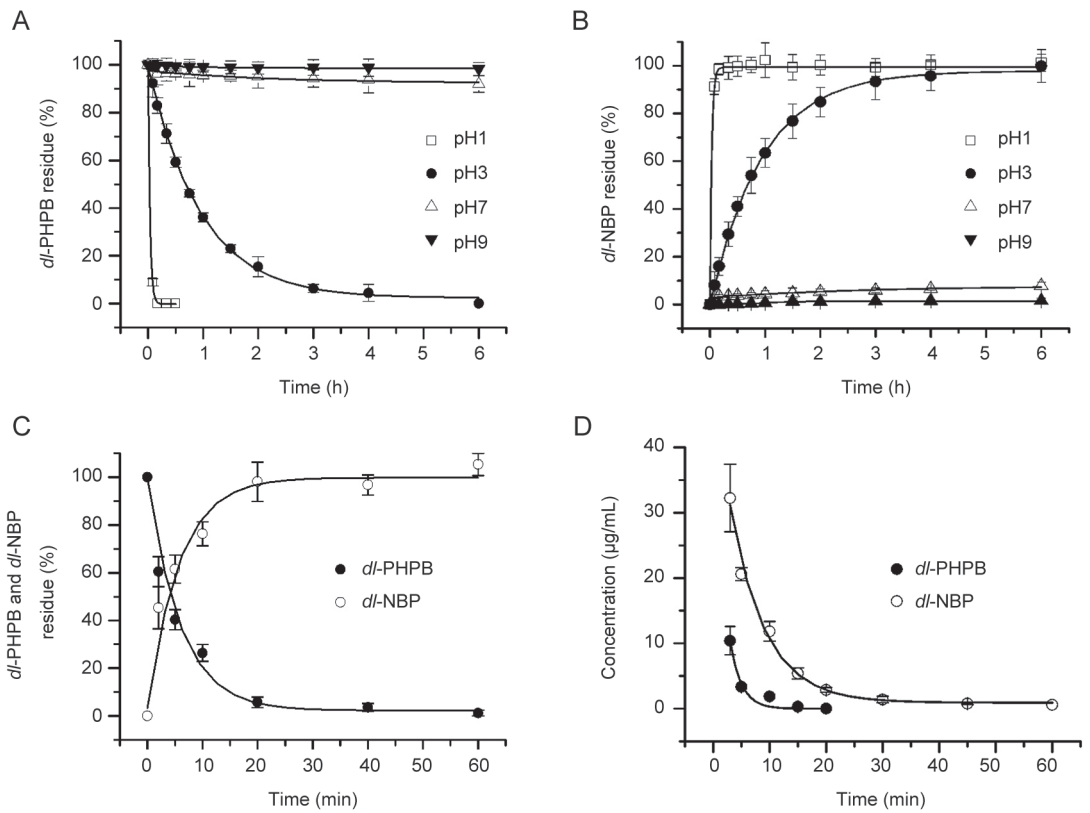


Figure 2. Time profile of *dl*-PHPB conversion. Time profiles of *dl*-PHPB and *dl*-NBP concentration in different pH solutions (A: *dl*-PHPB, B: *dl*-NBP) and SD rat plasma (C) were obtained after incubation at a final concentration of 10 μg/mL for different times at 37 °C (each point represents the mean±standard error of triplicate determinations). *In vivo* conversion (D), the mean plasma concentration versus time profiles of *dl*-PHPB and *dl*-NBP were obtained after the intravenous administration of *dl*-PHPB (20 mg/kg) in SD rats (n=6, 3/sex). Each point represents the mean±SD.

Table 1. The apparent first-order rate constants (K) and half-lives ($t_{1/2}$) of *dl*-PHPB conversion in solutions with different pH and SD rat plasma at 37 °C. n=3. Values are mean±SEM.

		K/h^{-1}	$t_{1/2}$/h	Received residues (%) after the 4 h incubation	
				dl-PHPB	*dl*-NBP
pH	1.0	150.30±5.69	4.59±0.59 (×10^{-3})	0.00±0.00	102.31±3.52
	3.0	1.02±0.14	6.76±0.45 (×10^{-1})	0.00±0.00	99.25±5.37
	7.0	2.19±0.54 (×10^{-3})	3.15±0.32 (×10^{2})	93.03±4.71	6.86±7.12
	9.0	8.56±0.74 (×10^{-5})	8.06±0.85 (×10^{3})	96.37±3.24	4.78±6.32
SD rat plasma		8.22±0.81	8.39±0.88 (×10^{-2})	0.00±0.00	98.96±5.77

Table 2. Mean values for the pharmacokinetic parameters of active metabolite (*dl*-NBP) following intravenous administration of *dl*-PHPB and *dl*-NBP in Beagle dogs and SD rats (n=6, 3/sex) .

		Beagle dogs				SD rats			
		dl-PHPB			*dl*-NBP	*dl*-PHPB			*dl*-NBP
		2 mg/kg	4 mg/kg	8 mg/kg	6.2 mg/kg	5 mg/kg	10 mg/kg	20 mg/kg	15.6 mg/kg
A	μg/mL	28.05	10.05	25.37	16.27	15.74	29.59	47.33	44.73
α	min^{-1}	1.05	0.22	0.15	0.11	0.34	0.16	0.16	0.20
B	μg/mL	0.80	4.64	1.02	3.63	4.47	1.54	1.47	1.89
β	min^{-1}	0.07	0.06	0.02	0.05	0.06	0.02	0.02	0.02
$V_{(c)}$	L/kg	0.69	1.36	0.76	1.05	0.62	0.80	1.03	1.05
$t_{(1/2)\alpha}$	min	0.66	3.19	4.75	6.34	2.04	4.31	4.33	3.52
$t_{(1/2)\beta}$	min	10.49	11.39	31.29	14.44	11.96	33.40	44.76	43.95
K_{21}	min^{-1}	0.09	0.11	0.03	0.06	0.12	0.03	0.02	0.02
K_{10}	min^{-1}	0.75	0.12	0.12	0.09	0.16	0.12	0.13	0.13
K_{12}	min^{-1}	0.28	0.05	0.02	0.01	0.11	0.03	0.03	0.06
AUC	μg·min·mL^{-1}	38.67	122.50	219.59	224.41	123.48	258.28	390.79	347.30
CL	L·kg^{-1}·min^{-1}	0.52	0.17	0.09	0.08	0.10	0.10	0.13	0.15

dl-PHPB. For this kinetic model, $t_{1/2}$ α and $t_{1/2}$β were 4.33 and 44.76 min, respectively.

Conversion of *dl*-PHPB was affected by esterase inhibitors

Due to the rapid conversion of *dl*-PHPB in rat plasma, the esterases mediating *dl*-PHPB metabolism were investigated by using specific inhibitors. As shown in Figure 3, in rat plasma, a nonspecific esterase inhibitor (NaF, 200 mmol/L) and metal ion chelating agents (EDTA-Na_2, 10 mmol/L and EGTA, 10 mmol/L) were highly effective inhibitors of *dl*-PHPB conversion (>90%). DTNB (arylesterase inhibitor, 1 mmol/L), PMSF (serine esterase inhibitor, 1 mmol/L) and BNPP (carboxylesterase inhibitor, 1 mmol/L) showed little or no suppression of the conversion of *dl*-PHPB. Interestingly, PMSF did not inhibit the degradation of *dl*-PHPB but was a relatively effective inhibitor of cholinesterase[21]. Donepezil (another cholinesterase inhibitor, CHEI) and teicoplanin (paraoxonase inhibitor, PONI) showed significant inhibition in concentration-dependent manners (P<0.05). These results indicated that paraoxonase was likely responsible for the lactonization of *dl*-PHPB to form its active metabolite *dl*-NBP in blood. This result was consistent with the inhibitory effects of NaF in rat plasma; 200 mmol/L of NaF was sufficient to inhibit PON activity[22].

Comparative plasma pharmacokinetics

The comparative pharmacokinetic studies of *dl*-PHPB and *dl*-NBP were carried out at equal-molar doses. The plasma concentration-time profiles of *dl*-NBP were determined following intravenous or oral administration of *dl*-PHPB and *dl*-NBP in SD rats and Beagle dogs. Figures 4A and 4B show that the concentration time curves of *dl*-NBP, whether converted from dosing *dl*-PHPB or from direct intravenous administration of *dl*-NBP, were nearly identical in dogs and in rats, implying a rapid transformation of *dl*-PHPB to *dl*-NBP in blood. The main pharmacokinetic parameters of *dl*-NBP after intravenous administration of *dl*-PHPB or *dl*-NBP are summarized in Table 2. The parameters were very similar at equal-molar doses, and the areas under the curve (AUCs) were nearly the same. However, after the oral administration of *dl*-PHPB, the peak concentrations and the AUCs of *dl*-NBP in both animal models were significantly higher than those after the oral adminis-

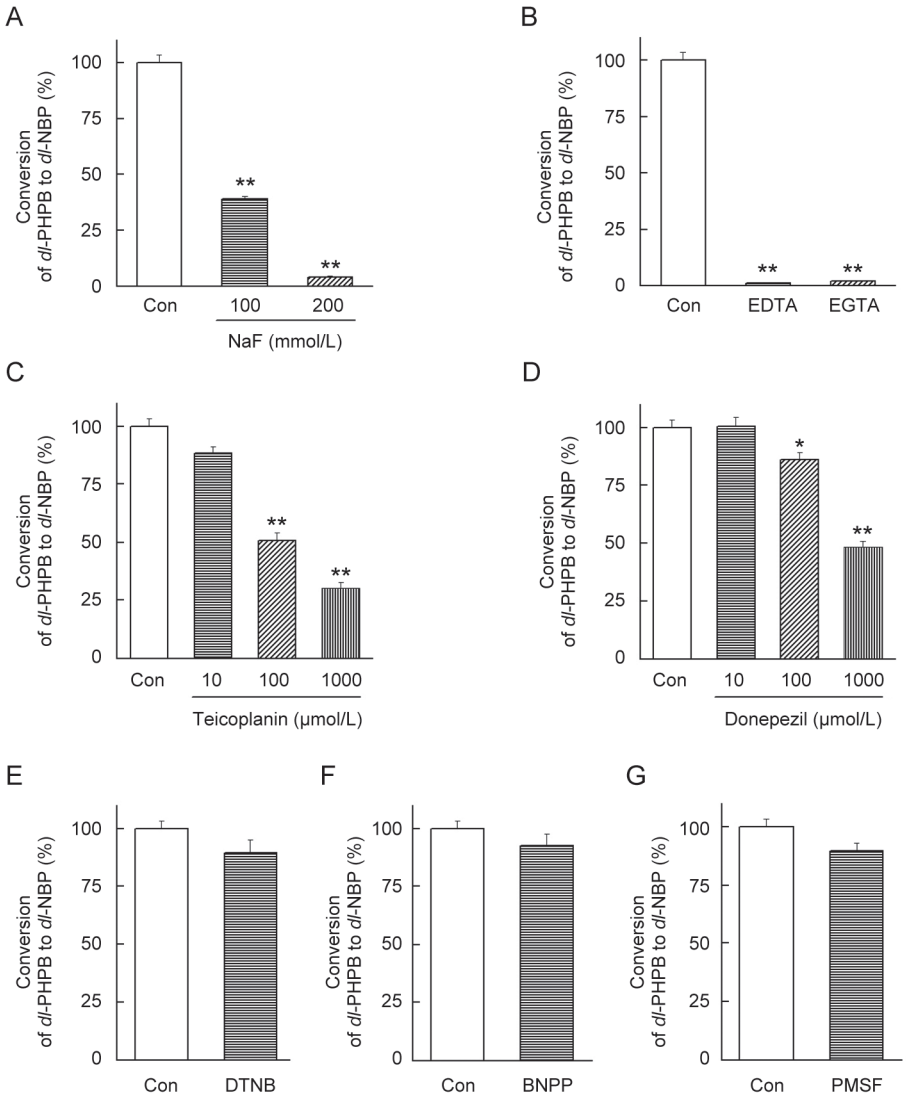


Figure 3. Effects of various esterase inhibitors on *dl*-PHPB conversion in rat plasma. The conversion of *dl*-PHPB to *dl*-NBP was highly effectively inhibited by NaF (200 mmol/L, a general esterase inhibitor) (A), and EDTA or EGTA (10 mmol/L, metal ion chelating agents) (B); partly inhibited by teicoplanin (1 mmol/L, a paraoxonase inhibitor) (C), donepezil (1 mmol/L, a cholinesterase inhibitor) (D); and negligibly inhibited by DTNB (1 mmol/L, an arylesterase inhibitor) (E), BNPP (1 mmol/L, a carboxylesterase inhibitor) (F) or PMSF (1 mmol/L, a serine esterase inhibitor) (G) (values are the mean±standard error of triplicate determinations). $^{*}P<0.05$, $^{**}P<0.01$ compared with control group.

tration of *dl*-NBP. This indicated that *dl*-PHPB was quickly absorbed and the AUC was higher than that of *dl*-NBP (Figures 4C and 4D). The main pharmacokinetic parameters of *dl*-NBP are listed in Table 3. At equal-molar doses, *ie*, 30 mg/kg of *dl*-PHPB and 23.4 mg/kg of *dl*-NBP in dogs and 200 mg/kg of *dl*-PHPB and 156 mg/kg of *dl*-NBP in rats, the time to peak (t_{peak}) was faster and the C_{max} and AUC were higher in the *dl*-PHPB groups (greater by approximately two-fold or higher) than those in the *dl*-NBP groups. Other parameters did not show any significant differences.

Dose-dependent pharmacokinetics were also studied. The plasma concentration time curves of *dl*-NBP in both animal models after the intravenous or oral administration of *dl*-PHPB are shown in Figure 5, and the pharmacokinetic parameters are provided in Tables 2 and 3. The data present a linear exposure value in the metabolism of *dl*-NBP in beagle dogs and a dose proportional exposure value in SD rats after intravenous or oral administration of *dl*-PHPB. We also tried to detect the plasma concentration time profiles of *dl*-PHPB. However, the conversion of *dl*-PHPB in plasma was too rapid, and the concentration was too low to be detected.

Distribution in tissues

Figure 6 shows the distributions of *dl*-NBP in rat tissues after intravenous or oral treatment with *dl*-PHPB or *dl*-NBP. The levels of *dl*-NBP were determined in heart, liver, lung, spleen, kidney, brain, stomach, fat, and small intestine tissues at different times after treatment. The results showed that the parent drug *dl*-NBP was primarily distributed in the stomach, kidney, fat, and brain tissues after either oral or intravenous

Table 3. Mean values for the pharmacokinetic parameters of its active metabolite (*dl*-NBP) following oral administration of *dl*-PHPB and *dl*-NBP in Beagle dogs and SD rats (*n*=6, 3/sex).

		Beagle dogs				SD rats			
		dl-PHPB			*dl*-NBP	*dl*-PHPB			*dl*-NBP
		10 mg/kg	30 mg/kg	100 mg/kg	23.4 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg	156 mg/kg
A	μg/mL	20.0	8.41	20.35	8.30	13.15	14.98	35.04	12.83
K_e	min^{-1}	0.06	0.01	0.01	0.01	0.06	0.04	0.06	0.02
K_a	min^{-1}	0.09	0.236	0.09	0.02	0.30	0.83	0.67	0.51
$t_{1/2(Ka)}$	min	7.89	2.78	7.37	33.14	2.31	1.10	1.10	1.35
$t_{1/2(Ke)}$	min	11.31	59.49	59.06	60.14	10.96	18.01	13.01	43.34
$T_{(peak)}$	min	13.55	25.29	13.41	63.47	6.58	3.60	4.01	6.96
$C_{(max)}$	μg/mL	2.64	6.83	13.23	1.79	6.84	14.35	26.04	11.13
AUC	$\mu g \cdot min \cdot mL^{-1}$	98.90	686.0	1518.00	323.50	262.80	478.00	1082.70	777.80
CL/F(s)	L/kg	0.10	0.02	0.02	0.03	0.08	0.08	0.06	0.03
V/F(c)	$L \cdot kg^{-1} \cdot min^{-1}$	1.70	1.25	1.68	2.65	1.28	1.79	1.24	1.50

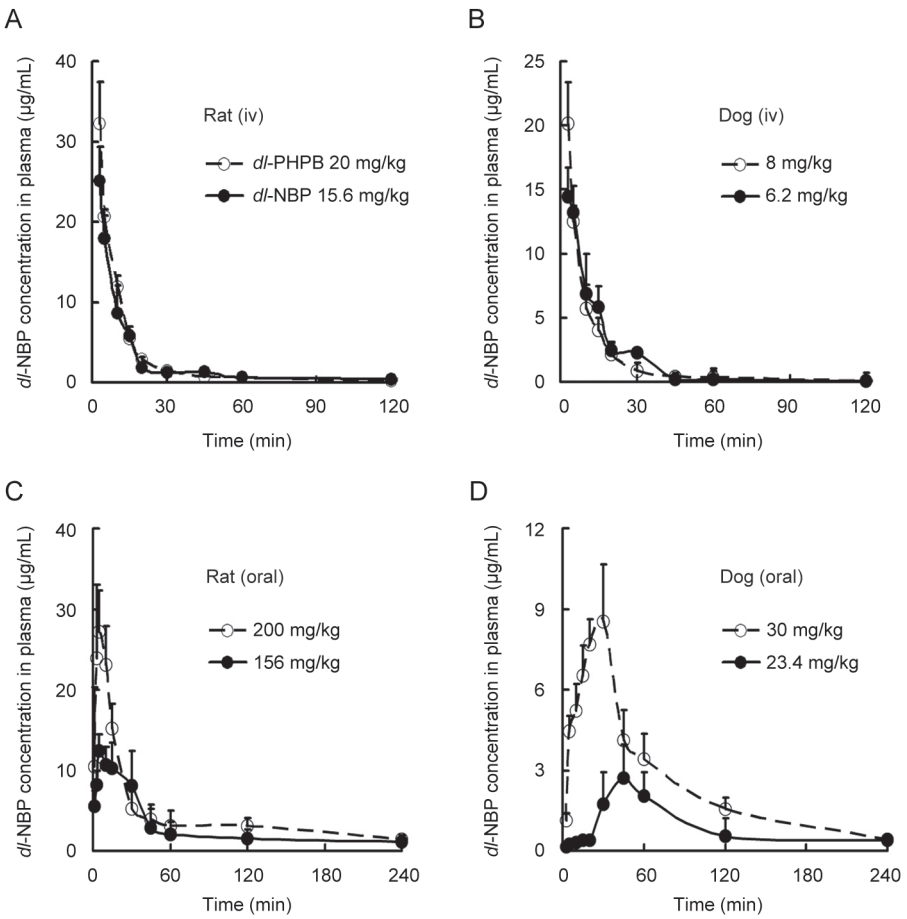


Figure 4. Comparative plasma concentration versus time profiles of *dl*-NBP after administration of *dl*-PHPB and *dl*-NBP. Based on the molecular weights of two compounds, *dl*-PHPB and *dl*-NBP were intravenously administered to SD rats (A: 20 mg/kg for *dl*-PHPB, and 15.6 mg/kg for *dl*-NBP) or Beagle dogs (B: 8 mg/kg for *dl*-PHPB, and 6.2 mg/kg for *dl*-NBP); and orally administered in SD rats (C: 200 mg/kg for *dl*-PHPB, and 156 mg/kg for *dl*-NBP) and Beagle dogs (D: 30 mg/kg for *dl*-PHPB, and 23.4 mg/kg for *dl*-NBP). Blood samples were obtained at 0, 1, 3, 5, 10, 15, 20, 30, 45, 60, 120, and 240 min after administration. Values are mean±standard deviation (*n*=6, 3/sex).

treatment with *dl*-PHPB or *dl*-NBP. The highest concentrations of *dl*-NBP appeared in the stomach and in fat after oral or intravenous treatment. In brain distribution, relatively higher levels were observed at different times after *dl*-PHPB treatment than after *dl*-NBP treatment, especially with oral administration; maximum concentrations of 40.6±8.9 μg/g at 60 min and 25.4±11.9 μg/g were observed, respectively.

Table 4. Cumulative recovery of *dl*-NBP (percentage of dose) in urine or feces within 72 h, after equimolar oral administration of *dl*-PHPB or *dl*-NBP in rats (*n*=6, 3/sex, values are mean±SEM).

Time	*dl*-PHPB (100 mg/kg)		*dl*-NBP (78 mg/kg)	
interval	Feces (%)	Urine (%)	Feces (%)	Urine (%)
4 h	0.32±0.03	0.07±0.02	0.21±0.01	0.02±0.00
8 h	0.90±0.40	0.12±0.04	0.48±0.01	0.03±0.00
12 h	1.77±0.56	0.20±0.05	0.82±0.01	0.18±0.11
24 h	2.17±0.72	0.36±0.10	1.13±0.04	0.23±0.11
48 h	2.43±0.70	0.46±0.12	1.16±0.04	0.25±0.11
72 h	2.44±0.69	0.47±0.12	1.40±0.04	0.25±0.10

Excretion of *dl*-PHPB

After equal-molar doses of *dl*-PHPB or *dl*-NBP by oral or intravenous administration, the cumulative recovery of *dl*-NBP in urine and feces of SD rats is shown in Tables 4 and 5. Less than 5% of *dl*-NBP was recovered in urine and feces after oral treatment of either compound within 72 h. *Dl*-PHPB was

Table 5. Cumulative recovery of *dl*-NBP (percentage of dose) in urine within 48 h, after intravenous equimolar administration of *dl*-PHPB or *dl*-NBP in rats (*n*=6, 3/sex, values are mean±SEM).

Time	*dl*-PHPB (10 mg/kg)	*dl*-NBP (7.8 mg/kg)
intervals	Urine (%)	Urine (%)
4 h	2.38±0.37	1.46±0.93
8 h	2.87±0.43	2.19±0.88
12 h	3.12±0.43	2.51±0.89
24 h	3.41±0.43	2.58±0.87
36 h	3.58±0.44	2.73±0.86
48 h	3.75±0.41	2.94±0.85

dl-NBP was undetectable in feces.

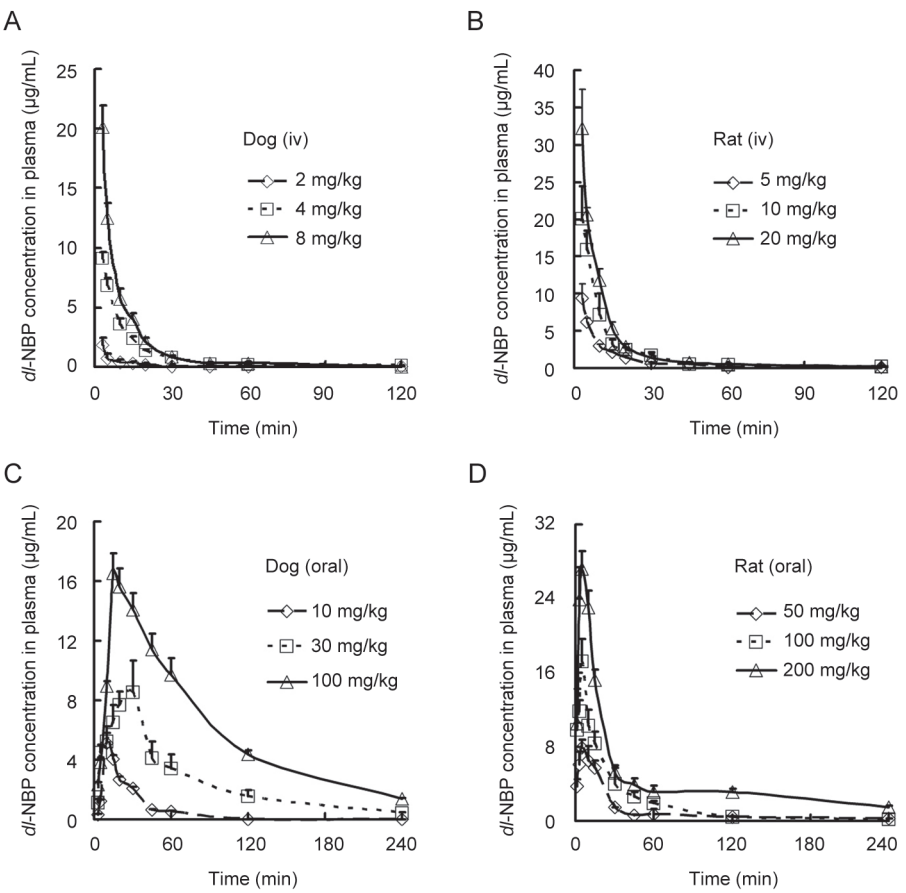


Figure 5. Mean plasma concentration versus time profiles of *dl*-NBP after the administration of *dl*-PHPB. *Dl*-PHPB was intravenously administered in Beagle dogs (A: 2, 4, and 8 mg/kg) and SD rats (B: 5, 10, and 20 mg/kg); and orally administered in Beagle dogs (C: 10, 30, and 100 mg/kg) and SD rats (D: 50, 100, and 200 mg/kg). Blood samples were obtained at 0, 1, 3, 5, 10, 15, 20, 30, 45, 60, 120, and 240 min after administration. Values are the mean±standard deviation (*n*=6, 3/sex).

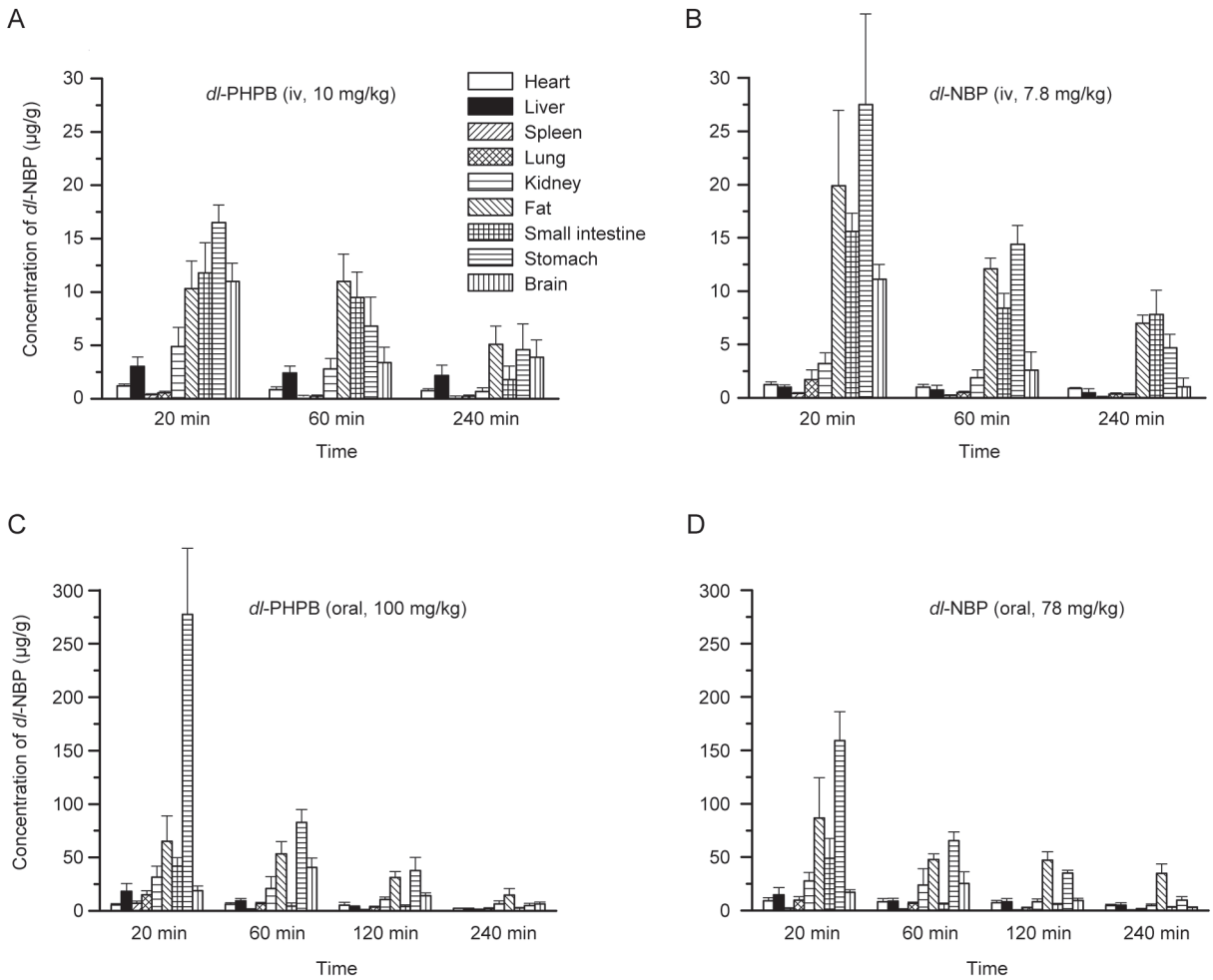


Figure 6. Tissue distribution of *dl*-NBP after equimolar dosing of *dl*-PHPB and *dl*-NBP in SD rats. The tissues were obtained at 20, 60, and 240 min after *dl*-PHPB (A: 10 mg/kg) or *dl*-NBP (B: 7.8 mg/kg) injection or at 20, 60, 120, and 240 min after oral *dl*-PHPB (C: 100 mg/kg) or *dl*-NBP (D: 78 mg/kg) administration. Values are the mean±standard deviation (n=6, 3/sex).

undetectable in the urine and feces of the *dl*-PHPB group. The excretion rates of *dl*-NBP in the urine or feces were similar between the two compounds, by either oral or intravenous administration, which implied that *dl*-PHPB was converted to *dl*-NBP and was then excreted as metabolites.

Discussion

The elucidation of the conversion mechanism of pro-drugs in chemical and biological samples is essential for enhancing pharmacological efficacy and safety. *Dl*-PHPB is liable to lactonization of the hydroxyl acid to form the pharmacologically active metabolite *dl*-NBP. The oral AUC value of the converted *dl*-NBP from *dl*-PHPB was two- to three-fold greater than that of *dl*-NBP directly administered.

Esters are typically unstable compounds *in vivo* due to esterases in bio-matrices[23]. Studies have identified *in vitro* hydrolyzing ester compounds in bio-samples that are involved in the *in vivo* metabolic pathway[24-28]. Non-enzymatic conversions indicate that chemical lactonization can occur. This pH-dependent conversion is important due to the various pH conditions encountered in the stomach (pH 2.0), bile (pH 8.5), plasma (pH 7.4) and intestines (pH 6.8). Because *dl*-PHPB is quite stable in alkaline conditions, *dl*-PHPB could be stored (could not be converted) in alkaline aqueous solutions.

The conversion rate was more rapid in rat plasma samples than in non-enzymatic solutions, indicating that enzymatic lactonization is the key mode of conversion in plasma. The conversion was markedly inhibited in rat plasma by NaF, a general esterase inhibitor[14, 22, 29], confirming that one or several plasma esterases are involved in *dl*-PHPB metabolism. Moreover, the conversion in rat plasma was almost completely inhibited by EDTA and EGTA (a general chelating agent for calcium) at 10 mmol/L and significantly restrained by donepezil (AChE inhibitor)[30] and teicoplanin (PON inhibitor) in a concentration-dependent manner[31]. However, PMSF, another inhibitor of AChE[21], did not show any inhibitory effects on *dl*-PHPB transformation. Additionally, due to the presence of calcium-dependent lactonase in sera[23, 32], EDTA and EGTA are potent inhibitors of PON and arylesterase via calcium ion chelation, which is essential for their hydrolytic activity[33,34]. Thus,

the results suggested that PON may be primarily responsible for the conversion of *dl*-PHPB in the plasma esterase.

The HPLC analyses of plasma samples after intravenous or oral treatment indicated a rapid and complete conversion of *dl*-PHPB to its active form (*dl*-NBP) in blood, which was consistent with the conversion *in vitro* studies (in non-enzymatic acidic solution or rat plasma). As described above, the conversion of *dl*-PHPB in plasma was too fast to be detected. After intravenous or oral dosing, the concentrations of *dl*-PHPB in plasma rapidly declined within 30 min, whereas *dl*-NBP concentrations remained at higher levels until 240 min. Thus, the metabolism of *dl*-PHPB, such as plasma pharmacokinetics, tissue distribution, and excretion, were carried out on its active form (*dl*-NBP) of *dl*-PHPB in SD rats and Beagle dogs. Compared with the oral administration of equal-molar doses of *dl*-NBP, the total drug exposure expressed as AUC and the C_{max} of the converted *dl*-NBP were two-fold higher in SD rats and Beagle dogs after the oral administration of *dl*-PHPB. We also found that the pharmacokinetic parameters of *dl*-NBP following intravenous administration of *dl*-PHPB in rats and dogs were similar to those of *dl*-NBP based on equal-molar dosages. Thus, the pharmacokinetic profile of *dl*-PHPB showed a rapid absorption, and the converted *dl*-NBP showed a higher bioavailability.

The distribution study showed that the concentrations of *dl*-NBP were low or undetected in all tissues after 240 min, indicating no long-term accumulation after oral *dl*-PHPB administration. However, there was a relatively higher distribution in fat tissue than in *dl*-NBP-treated animals after intravenous dosing of *dl*-PHPB. These effects may help to maintain plasma drug concentrations and may result in higher bioavailability. When *dl*-PHPB was orally administered, the distribution of converted *dl*-NBP in rat brain was enhanced by 60% compared with that observed in oral equal-molar doses of *dl*-NBP. The high distribution in the brain confirmed the pharmacological effects of *dl*-NBP on anti-cerebral ischemia[1, 5–7] and the therapeutic benefit of *dl*-PHPB.

Our study showed that no original form of *dl*-PHPB was excreted and only less than 5% of *dl*-NBP was recovered in the urine and feces after the administration of *dl*-PHPB in SD rats within 72 h. Because of the rapid and complete conversion of *dl*-PHPB to *dl*-NBP, this may indicate that the converted *dl*-PHPB to *dl*-NBP in blood or tissues was primarily excreted in the parent-drug form. A low percentage of the parent-drug form was detected in urine and feces. Similar observations have been reported in rats after the administration of *dl*-NBP[35, 36]. Within 24 h following oral [^{3}H]-NBP administration, the total radioactivity in urine and feces was 73.1% of the original dose, and the total prototype drug excreted in urine and feces was 2.53% of the dose[36]. Furthermore, 23 metabolites of *dl*-NBP were detected in human plasma and urine[37]. Thus, the metabolic pathway of *dl*-PHPB *in vivo* may be similar to that of *dl*-NBP. Additionally, *dl*-PHPB is a weakly basic drug, exhibits pH-dependent stabilization and is susceptible to gastric and intestinal (GI) tract pH change. The varying GI pH levels may also contribute to differences in drug absorption. The rate of gastric acid secretion is known to be widely different among species. For example, rats and humans have good acid secretions but dogs have poor secretions[38-41]. In the present study, the results showed that the bioavailability of *dl*-PHPB in dogs was higher than that in rats. The differences in bioavailability between dogs and rats are most likely due to low basal gastric acid secretions increasing GI pH and enhancing the stabilization of *dl*-PHPB in this species. Further studies are required to clarify the effects of pH changes on the metabolism of *dl*-PHPB, conversion mechanisms, metabolic pathways for *dl*-PHPB, and potential interactions with other drugs.

In conclusion, as a pro-drug, *dl*-PHPB was rapidly and completely converted into *dl*-NBP after treatment in rats and dogs. The C_{max} and AUC values of *dl*-NBP converted from *dl*-PHPB were significantly higher than those from direct *dl*-NBP administration. Conversion *in vivo* was primarily mediated by plasma esterases. The active form was more distributed in fat and brain tissues. The *dl*-PHPB metabolic pathway after conversion may follow that of *dl*-NBP.

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Author contribution

Xiao-liang WANG conceived and designed the experiments; Jiang LI, Shao-feng XU, Nan FENG, and Ling WANG performed the experiments; Jiang LI wrote the paper; Xiao-liang WANG, Jiang LI, and Ying PENG analyzed the data.

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